

A major museum goes 'populist'

Yet another British institution is in the throes of ill-calculated change: the British Natural History Museum will subordinate research to popular exhibition. It should rather have rejected the task imposed on it.

ONE of the simpler moral tales put out by the classical Greek writer Aesop is that of a dog carrying a bone in its mouth when crossing a stream that chose to grab at the reflection of its bone in the water beneath, losing both what it had and what it sought to have. The moral is that greed can lead to deprivation. Nobody can accuse the British Natural History Museum (NHM), which has been having a rotten time, of that old vice, but the dilemma in which it finds itself is much like that of Aesop's dog.

The museum's paymaster, the British government, has kept it on a shoestring, with a budget declining in real terms for the past five years. Like other public museums and art galleries, the NHM has been compelled to charge admission fees and to raise external funds in other ways, increasing the turnover of its retail shop for example. Its first 'corporate plan' promises even greater efforts in this direction. Meanwhile, to make room in the annual budget for new ventures, including more arresting public displays, the museum has decided that 55 tenured posts should be shed, some of them being replaced by short-term appointments. (see page 4). The danger is that the museum's academic reputation and future research programme will be sacrificed for a future as a temple of public understanding that is illusory.

Stimulant

The world best knows NHM as Britain's primary research museum. Its collections of fossil material and of zoological and botanical specimens have been and remain of immense importance for taxonomists (palaeontological and otherwise) and for biology more generally. As national research institutions must, NHM includes researchers among its staff, partly so as to preserve expertise in the use, extension and management of an important collection and partly so that the pursuit of important research themes should not be entirely at the mercy of the necessarily shifting interests of academics. But NHM is also, as it should be, well-known in Britain as a stimulant of youthful enthusiasm for fossils and for the grandeur of the evolutionary concepts which they imply. The museum is also blessed (but financially encumbered) by a nineteenth-century building which comes close to being "a museum that should be in a museum" — the poet Dylan Thomas's affectionate put-down of his Welsh home town's museum. (Ironically, that museum is to be closed this summer for lack of funds.)

The troubles at NHM go back for decades, so cannot be laid at the door of the present management or even the present government. During the 1950s and 1960s, when the pattern of research elsewhere was being transformed, the museum gave the impression of being deeply asleep. Perhaps hampered by the fact that its full-time researchers are essentially tenured civil servants, it never had the wit to find ways of recruiting a stream of graduate students on its own account (although plenty from elsewhere use its collections), while the supply of short-term research posts has been pitifully small (and shrinking). Increasingly since the early 1970s, the museum's staff has been shockingly paid and even less able than, say, colleagues at British universities to attend important scientific meetings. Successive managers have allowed circumstances (chronic penury) to decree that the museum's research should take on a musty flavour. Who can now be surprised that a new management has taken the knife to it? Thus do bad decisions often justify themselves. Yet the eye-catching banality of the new management's chosen research themes is less readily forgiven. (Taxonomy and evolution, including cladistics, are presumably subsumed in "biodiversity", but what expertise has the NHM in "human health"?)

Even so, many of the elements of the corporate plan are sensible, even enlightened. For example, it is planned to distinguish between the essential museum skill of curatorship and the practice of scholarship and research. Taking the steady squeeze on funds as a given, some such accommodation is probably unavoidable, even though it remains to be seen whether it will be possible to recruit able curators who are denied the opportunity of research. The obvious snag is that the museum's own research function, subordinated as it will be to what the plan calls "front of house" — there is a chilling promise that the research programme will be "responsive to changes in audience needs" — will be the first to suffer when there is a further tightening of budgets. The betting is that this distinguished research institution will face continuing attenuation of influence in research.

The metaphor of Aesop's dog is therefore in some ways misleading; the bone it carries is shrinking anyway. What of the reflection in the water? NHM's reputation as a self-conscious purveyor of public understanding of science goes back to the late 1970s, and to an elaborately designed, widely popular, but unsurprising and even simplistic



exhibition on cell biology in which the museum's collections played no essential part. That dubious success seems to have gone to the museum's head. Professionally mounted exhibitions have become central to its work. (Of such a one, in 1981, this journal found it necessary to complain that the museum was telling an ambiguous tale on evolution, as if it were sited in Tennessee or Louisiana — see *Nature* **291**, 373; 1981.) Since then, and especially since the introduction of admission charges in 1987, the museum has become a more skilled and circumspect exhibitor. How else to stimulate demand? What the plan says is that there will now be more of that, despite the decision that exhibition staff should share in the job cuts.

This is where the dilemma bites. Nobody will deny that countries such as Britain need better public understanding of science, or that the prospectus now issued will be valuable to that end. A sufficiently tough-minded management may also be able to persuade its visitors that technical activities are not merely a threat to public health and the environment (the soft options), but such exciting and beneficial ways of transforming natural conditions that declining recruitment to science studies will be reversed. But there is no reason why the NHM research programme should be sacrificed for this purpose, however admirable. By using its ingenuity to live within its arbitrarily decreed budget, the new management at the NHM has allowed itself to be forced into a false position.

That is the essential error of what is fashionably, but laughably, called the corporate plan. If put forward to an agency other than a government committee, last week's document would excite only derision. At one stage it boasts that "income-generating activities" have increased to 25 per cent of the total budget, and are due to increase to 30 per cent in the next five years. But these are gross figures, and take no account of overheads. This year, for example, the museum expects to make a profit of £400,000 on sales of £2.5 million at its shops, but without taking account of costs such as occupancy, heat and light. The museum would do better to disencumber itself of management tasks for which it has little flair by selling a concession to a commercial organization, as airports do.

Shabby

That is but one reason why the museum's management should be ashamed of its shabby document. There can hardly ever have been a piece of paper about the future of a great scientific institution so devoid of scientific content. The fear is that the future pattern of its public work will similarly be content-free. Thus the plan says that "with the new and increasing concern about man's effect on the global environment, the Museum's knowledge and expertise are of greater relevance than ever before". Really? That claim may carry some weight with the Office of Arts and Libraries, but the taxonomists who make up NHM's constituency will be entitled to a hollow laugh.

What has happened and is happening to the NHM is what has happened to other British institutions faced with shortages of funds in the past decade, the British univer-

sity system for example. Able and intelligent people have been persuaded to administer them in ways that offend their purpose. The managers find themselves in the position of trusted prisoners in most jails, tricked into believing that they will do the dirty work more sympathetically and sensitively than can the jailers. Aesop's dog's dilemma is, in this case, the more piquant because both the bone and its reflection are worth having: Britain needs both a centre for taxonomic research and the superb general exhibition of science. There would have been a case for a reassessment of the research programme, and for appropriate reforms, as well as for a less self-congratulatory essay in public exhibition. But the museum should have firmly told the government that it cannot have both functions for the price of one, especially in a building in which the cost of maintenance will this year amount to a third of the total budget. □

Arms control hiatus

The prospects for agreements on strategic arms and conventional forces in Europe have recently dimmed.

1990 MAY not, after all, be the wonder year for arms control. Both the talks on strategic arms at Geneva and those on conventional forces in Europe at Vienna are said by sources in the West to have run into Soviet inflexibility of a kind that has been unfamiliar for several months. That development is understandably disconcerting, but not surprising. If Soviet unwillingness to make the last few compromises is more than a tactical manoeuvre, the explanation probably lies in Soviet misgivings about the uncertain outlook for European security as a whole.

The obvious stumbling block is the future of the formal European alliances, the North Atlantic Treaty Organization (NATO) and the Warsaw Pact. Mr Mikhail Gorbachev, by insisting that the formal right of Eastern European states to self-determination should be taken seriously, has made possible the changes in Central Europe that have dominated the past few months. But now he is faced with the determination of East Germany to unite with West Germany constitutionally, and the prospect that the reunited country will remain a member of NATO. Seen from Moscow, this is an uncovenanted setback, which will be resolved only when a new framework for European security has been devised. Western governments have been slow to appreciate the need for a resolution of this difficulty, without which German reunification can hardly be more than a formality. It is true that the issue may be clarified in June, when there is to be a conference at Vienna within the framework of the Helsinki agreements of 1978, but it will be a shame if the arms control agreements also have to wait until there has been progress in those talks. Should not Western governments say now, in advance of what are certain to be protracted negotiations, what changes they have in mind? The best course would be an amalgamation of the two alliances. □

Hubble Space Telescope takes first shaky steps

■ Long-delayed launch is perfect

■ High-strung computers make NASA nervous

Greenbelt, Maryland

AFTER a near-perfect launch last week, the Hubble Space Telescope (HST) has proved unexpectedly difficult to control and may remain partially crippled, National Aeronautics and Space Administration (NASA) officials said. Of greatest concern are the two large high-gain antennas that HST uses to communicate, via relay satellites, to the ground. By early this week, one antenna seemed to be working normally, but the other appears to be obstructed by a misplaced one-inch cable. The antenna is unable to move through its full rotation without triggering sensors that detect excessive force.

Normally, NASA engineers would disable the faulty antenna and rely on the good one, but HST's two antennas are designed to move together; it is impossible to send commands to one and not the other. Although engineers have been able to lock the troublesome antenna, it continued to "dither" when the other was moved. Even if the two antenna can be reprogrammed to operate independently, the body of the telescope would at times block the path between the working antenna and the link satellites, interfering with the transmission of data.

Instead, HST controllers plan to keep both antennas in operation, but limit the

can be sorted out. Bringing HST out of safe mode takes time, even when the malfunction is relatively benign. In the mild safe-mode level, communications links remain open, but the telescope will not move any of its parts. In more severe shutdowns, even communications are reduced.

Last Friday (27 April), as controllers prepared to open the hinged 'lens cap' that protects the 94.5-inch primary mirror, an error signal from the obstructed antenna put HST into its most shallow safe mode. The antenna was turned off and HST was brought back to life to allow the lens door to be opened, but controllers discovered that the process of opening the door jostles the entire telescope, causing motion-sensing gyroscopes to record an unanticipated move. Assuming that the sensors were in error, HST's main computer temporarily turned off two of the four main gyros; without at least three gyros working, HST cannot manoeuvre.

The telescope responded to the loss of the control gyros by dropping into a deeper safe mode. After several tense hours, NASA engineers were able to coax HST back into operation. But a top priority will now be to adjust the sensors or reprogram the main computer to avoid such a painful routine every time the door is opened or closed. Many of HST's components, the door among them, could not be adequately tested on the ground under the force of gravity.

Once the aperture door was open, the shuttle *Discovery*, which had been flying in tandem with the telescope in case of emergency, was free to return to Earth. Astronauts had been on board and prepared to repair the solar panels, the door, or certain other parts that could have malfunctioned. But the rogue cable was not detected until Sunday, when NASA engineers examined the video images taken as HST was released by the shuttle. By that time *Discovery* was already on its way home.

Because of HST's teething pains, the first out-of-focus pictures from the telescope will be delayed by nearly a week. Calibrating and testing the instruments will take another six to eight months. Only then will scientific observations begin, although some detailed pictures should be available within a month.

G. Christopher Anderson

CZECHOSLOVAKIA

Academy law due for welcome reform

Prague

PROFESSOR Armin Delong, the newly appointed chairman of the Czechoslovak State Commission on Science and Technology, seems to have won a concession for which the scientific community has been asking for some time — the promise of early legislation to amend the law that governs the Czechoslovak Academy of Sciences.

At a meeting with the chairman of the federal assembly, Alexandr Dubček, a delegation of scientists led by Delong won agreement that there will soon be *de jure* recognition of the changes in the organization of the academy the federal government has introduced.

The sensitive issue of membership of the academy was also discussed at the meeting. Despite pressure from the scientific community for members appointed for strictly political reasons to resign, only one has so far done so. It was agreed that the membership should now be dissolved, and that new members should be elected on a federal basis. That, acting vice-president Katčtov explains, will cause less pain.

Among other issues raised with Dubček were the problems of the proposed competitive grants system and the general fear that younger badly paid scientists will leave the country. The delegation hopes that Dubček's parting words — "we shall be needing you more than you need us now" — are a sign of the government's goodwill towards science.

Delong's appointment, to replace 52-year-old veterinarian Dr František Reichel, has been widely welcomed. Reichel, who is a prominent member of the Catholic Peoples' Party, was largely unknown before taking office earlier this year. Delong, 65, is both a vice-president of the academy and director of its Institute of Scientific Instruments at Brno. He is widely respected for his work in electron optics and lithography, as an organizer and as a spokesman for reform. As chairman, he is expected to accelerate long-awaited changes at the commission of which he is now the head.

Veronika Maxová

UK CHIEF SCIENTIST

Greener advice?

London

PROFESSOR Bill Stewart, secretary of the Agricultural and Food Research Council, will be the government's next chief scientific adviser, replacing Sir John Fairclough. The appointment will be seen as a sign of prime minister Margaret Thatcher's much-vaunted interest in environmental issues. Stewart serves on the Royal Commission for Environmental Pollution and is a former member of the Natural Environment Research Council.

Peter Aldhous

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

HST floats free of *Discovery*'s robot arm. (AP). rotation of the obstructed one to about 140°, 40° less than planned, to stop it hitting the stray cable. Short of another shuttle mission and a spacewalk, there is no way to remove the cable. The reduced antenna travel is expected to complicate data transmission and increase the time needed for some experiments.

A continuing difficulty is that when the cautiously programmed HST detects serious errors or unexpected motions, it goes into a "safe mode" in which certain systems are shut down until the problem

Taxonomy pays for bad image

London

RESEARCHERS at the Natural History Museum (NHM) in London went on a one-day strike on 24 April to protest against the museum's controversial 1990–95 corporate plan, which proposes the loss of 51 out of 300 research and curatorial posts during the next two years. Many of the tenured posts are to be replaced with short-term fellowships (see *Nature* 344, 805; 26 April 1990), a move that will improve the NHM's financial health but may threaten its standing as a taxonomic research centre. On 26 April, the researchers resolved to strike again tomorrow (Friday, 4 May) if the museum's director, Neil Chalmers, refused to withdraw the plan.

Scientists at other UK museums are concerned at the damage that might be done by the new plan. Taxonomic research, in which the NHM is pre-eminent, is "deeply unsexy", according to Simon Conway Morris of the University of Cambridge, but is the "bedrock" of all biological research, and in the light of concern over decreasing global biodiversity the cuts come at "just the wrong time". Andrew Knoll, of the Botanical Museum of Harvard University, finds it "a little

grade, and promotion will be possible only when senior staff depart. The danger is that young curators will leave rather than wait, which would undermine the museum's tradition of expertise founded on long service.

By abolishing tenure in favour of short-term contract work, Chalmers will reduce the NHM's wage bill while increasing flexibility. But university-style fellowships may be inimical to museum research, built on the continuity of expert knowledge acquired over the course of a career. "Museums provide a unique environment suitable for specimen-based, non-applied research which is too often ignored by other institutions", says Philip Currie, of the Tyrrell Museum of Palaeontology in Drumheller, Alberta, and "there's no point in doing what the universities already do quite well", adds Conway Morris. But Chalmers points to the Smithsonian Institution and the American Museum of Natural History in New York as institutions with traditions of short-term as well as permanent staff.

Chalmers hopes, by concentrating research under six general headings — biodiversity, human origins, human health, environmental quality, living resources and mineral resources — to create peaks of excellence that cancel the troughs left by the research cuts. Gone are the days, he says, when NHM could hope to cover all areas of the natural world in equal depth. But Currie criticizes the choice of subject area as "catchy buzz-words", and asks "are these not functions normally performed by government departments, universities and consulting firms?". Critics feel that Chalmers has failed to comprehend that the kind of research to be found at the NHM is unique. Cuts were bound to attract criticism, but some seem particularly inappropriate given the areas in which excellence is meant to be maximised. The Parasitic Worms section does work of potential economic importance, but its staff of six is to be halved, and the *Host-Parasite Catalogue*, a database started in 1922, is to be abandoned despite work to computerize it over the past two and a half years.

Responsibility for the NHM passed from the Department of Education and Science (DES) to the Office of Arts and Libraries (OAL) in July 1987. An OAL spokesperson said that contact between OAL and the museum before submission of the new corporate plan was "informal". Chalmers says that the OAL "has been very understanding and sympathetic to our scientific activities... we're in regular contact with OAL officials", but on the question of whether the OAL seeks to influence NHM policy, "the answer is clearly 'no'".

Henry Gee

Seven more nations nearly 'nuclear'

Washington

THE 1980s were a bad decade for nuclear proliferation, with several nations developing or expanding nuclear weapons programmes, according to a report from the Carnegie Endowment for International Peace. The report found loopholes in the nuclear export controls of several industrialized nations, but found most fault with West Germany, calling it "the weak link" in the multilateral export control system."

The report says that seven nations — Argentina, Brazil, India, Iraq, North Korea, Pakistan and South Africa — have all engaged in nuclear smuggling or have bent existing export requirements to build their nuclear weapons programmes. Discussion of Israel's nuclear programme is largely absent from the report, ostensibly because it was already in place by 1980.

The seven nations are still several years away from producing nuclear bombs, but Israel, India, Pakistan and South Africa are termed "*de facto* nuclear powers". None of these nations has formally acknowledged the existence of a nuclear programme despite the fact that many incidents involving clandestine and quasi-sanctioned trade in nuclear material and technology have been documented.

The report calls upon the United States and other advanced nations to penalize states that repeatedly engage in nuclear smuggling through the threat of economic sanctions.

Seth Shulman

* *Nuclear Exports: The Challenge of Control*, by Leonard S. Spector, Carnegie Endowment for International Peace, Washington DC, April 1990.

PUBLIC HEALTH

Radon scare doubted

London

A group of physicists from the University of Bristol claim, in last week's issue of the *Lancet*, that radioactive radon gas seeping into buildings may cause leukaemia and several other cancers. The team, led by Denis Henshaw, correlated leukaemia records with average indoor radon concentrations across 14 countries. They believe that radon becomes concentrated in fat cells in bone marrow.

Michael O'Riordan, head of the National Radiological Protection Board (NRPB)'s radon programme, believes the Bristol group may have "attached too much significance to their hypothesis". Exposure to radon gives a radiation dose in the lungs 100 times that in the red bone marrow. Even if the Bristol hypothesis is correct, O'Riordan says that only about 20 out of 2,800 deaths a year from myeloid and acute leukaemia may be linked to radon. The NRPB estimate that 2,500 lung cancer deaths a year in the UK may be radon-induced.

Peter Aldhous

REDUNDANCIES AT THE NATURAL HISTORY MUSEUM

Department (posts lost)	Breakdown
Botany (7)	Research on bryophytes and diatoms 4 Curation of algae & herbaria.....2 Taxonomic computing1
Entomology (17)	Research on insect sounds, beetle functional morphology, weevils, mayflies, lacewings, caddis flies, heteropteran & cocoidean bugs, bees, wasps & microlepidoptera12 Curatorial reorganization5
Mineralogy (10)	Research on mineral structure, building stone and gems4 Database production & publication1 Curation: collection management4 Electron microscopy1
Palaeontology (10)	Research on fossil birds, plants, mammals (except primates) & Tertiary molluscs6 Curation of fossil invertebrates3 Editorial work on Museum Bulletin.....1
Zoology (7)	Research on archaeozoology, testate amoebae, histology, parasitic worms 5 Curation of molluscs & lower vertebrates2
Total loss of science posts — 51	

sad" that the study of biodiversity in the United Kingdom is thought so marginal that the NHM will close departments "in which they have been major contributors". Ken Joysey, curator of the Museum of Zoology at the University of Cambridge, condemned the cuts as "ludicrous".

The cuts apply to posts rather than people: researchers can, in some cases, apply for positions in the new system, which is intended in part to foster curatorship as a profession with a career structure. But in the plan as now proposed, the career opportunities are limited because each post will carry a specific civil service

UK RESEARCH SPENDING

Playing the numbers game

London

A REPORT from the House of Lords select committee on science and technology charges that official government figures for British research spending paint a misleadingly favourable picture, and concludes that "as a nation we are investing too little in civil R&D and the situation is getting worse".

The UK defence research and development (R&D) budget, at £2,300 million, accounts for half of Britain's total R&D effort, a proportion exceeded only by the United States. But the Lords report, "Definitions of Research and Development", argues that up to half of this money goes to product development rather than innovative work. Internationally accepted definitions used by the Organization for Economic Cooperation and Development exclude product development work from R&D accounting.

The report also shows how ministers quote carefully selected statistics to compare British research spending with that of other nations. If, as the committee recommends, expenditure is expressed as a percentage of gross domestic product (GDP), and if defence spending is removed, British R&D spending comes out below West Germany, France and Japan, but above the United States. And if private sector spending is included, Britain falls behind all four nations.

Professor Denis Noble, from the University of Oxford and president of the Save British Science pressure group, says the position of British civil science is even worse than the "bald statistics" imply. Although the US government's civil science budget compares poorly to Britain's, using the select committee's indicator, Noble says that whereas "a very substantial fraction" of US military research spending gets through to the civil sector, almost all British defence research is done 'in house' or contracted to defence procurement companies.

The select committee's report singles out the weakness of private sector research for particular criticism. But an international survey of government-supported academic research, summarized in the next issue of *Science and Public Policy*, provides a similarly gloomy analysis of British government spending.

Ben Martin, John Irvine and Phoebe Isard, from the University of Sussex, found that British government spending in the seven years up to 1987, at around 0.4 per cent of GDP, averaged only four fifths of that in France, West Germany and the Netherlands. The US and Japanese governments spent a smaller proportion of GDP on academic research even than Britain. But these countries have large private university sectors, so may be less

relevant for comparison with the UK. The study was paid for by the UK Advisory Board for the Research Councils (ABRC) and the US National Science Foundation (NSF), and updates an earlier study funded by the ABRC (see *Nature* 323, 591; 1986).

British government support in 1987 for environmental science, mathematics, computing and engineering compared favourably with France, West Germany and the Netherlands. But the Sussex team calculate that an extra \$500 million should have been spent in 1987 to bring UK spending on physical and life sciences, as a proportion of GDP, in line with the other three European nations surveyed. Increases to the British science budget in 1988 and 1989 may have remedied this deficiency somewhat, but the French and German governments have also increased spending.

BRAZILIAN ECONOMIC CRISIS

Graduates see grants vanish

São Paulo

ABOUT 15,000 graduate students in Brazil are still waiting for their stipends, some 50 days after their last payment. Another 2,200 graduate students abroad are a little luckier and have received their April payments, but they are now worrying about their chances of receiving money for May. The nationwide evaluation of postgraduate courses has been abandoned.

These are some of the effects of Brazil's new economic austerity measures and the reform of federal bureaucracy. The innocent victim was a Ministry of Education grants agency known as the Coordinating Agency for Advanced Training of High-Level Personnel or CAPES. Last week, the agency was not coordinating much besides its own survival.

CAPES was simply forgotten by the planners of the administrative reform, and was accordingly considered extinguished by a provisional measure, number 150. The Minister of Education, Carlos Chiarelli, tried to recreate the agency, but could do nothing immediately as action was required by Congress.

An amendment to measure 150 was finally passed and CAPES was reborn. But in the meantime, students received no money and postgraduate programmes had no coordinating body.

As a protest, graduate students at the University of São Paulo took to begging money from drivers at traffic lights at the campus. But CAPES new directrice, anthropologist Eunice Ribeiro Durham, says that funds will be available again soon. Durham is a former vice-president of the Brazilian Society for the Progress of

Sir David Phillips, chairman of the ABRC, confirms that the survey's results will be used as a basis for discussions with the Department of Education and Science on the size of next year's science budget. Ministers may argue that Britain does not need to spend a similar proportion of GDP to other European countries to maintain a competitive civil science base. But a second paper in the same issue of *Science and Public Policy* shows that the British share of world output and citations of basic scientific research papers is declining.

In collaboration with Computer Horizons Inc. (CHI), from New Jersey, and Diamond Systems, a British company, Martin and Irvine surveyed the CHI/NSF Science Literature Indicators Database. Martin and Irvine conclude that "British performance is weak and deteriorating in several scientific areas . . . important for future industrial competitiveness", including solid state physics, polymer chemistry and computer science. **Peter Aldhous**

Science (SBPC). That and her friendship with the Secretary of Science and Technology, Jose Goldemberg, give hope to researchers that help may actually arrive.

At the same time, the new economic measures are wreaking havoc among the scientific societies. The 36th Brazilian Genetics Society Meeting had to be cancelled last month because the society could not obtain the resources it had counted upon — the government has blocked access to 80 per cent of the money in all bank accounts, savings and investment accounts as part of its drastic economic plan.

The president of the Genetics Society, Francisco Moura Duarte, has had to send telegrams and letters to the 65 foreign researchers invited to the meeting. The meeting, scheduled for 28 to 31 May, also included the first workshop of the Latin-American Association of Environmental Mutagenesis, Carcinogenesis and Teratogenesis.

A failure of the National Council for Scientific and Technological Development (CNPq) to provide sufficient money was partly responsible for the cancellation of the conference. But Duarte is particularly angry with the federal government. The Secretary of Science and Technology, José Goldemberg, had promised to try to release the money held by the scientific societies from government restrictions. Duarte's frustration led him to say that the military presidents who ruled Brazil from a coup in 1964 till 1985 had been more "sensible" in their attitude towards science than the presidents of the democratic period. **Ricardo Bonalume**

Drug companies divided

Washington

PROPOSALS introduced in the US Congress last week may dilute the benefits to drug companies of the Orphan Drug Act by forcing them in some cases to "share" the temporary marketing monopoly that the Act permits. Representative Henry Waxman (Democrat, California), chairman of a health and environment subcommittee, has introduced two bills to 'fine tune' the Act, which at present provides drug companies with grants, tax credits and a seven-year-period of marketing exclusivity in the United States as incentives to develop drugs for diseases that affect fewer than 200,000 people.

Since its inception in 1983, 333 drugs have been issued with orphan drug status, and 45 have been approved by the US Food and Drug Administration (FDA). But congressional concern over the high prices of some profitable drugs protected from competition by the Act has prompted Waxman to take action. He believes that "in a small number of cases, the Act has provided monopoly protection for drugs that are very profitable and that would have been developed without the incentives of the Act".

Three drugs — human growth hormone, aerosol pentamidine (approved to treat an AIDS-related pneumonia) and erythropoietin (EPO, approved to treat anaemia in patients with chronic renal failure) — cause particular concern. Hearings were held in February to consider whether orphan status for these drugs had resulted in unwarranted monopolies and unnecessarily high prices.

Perhaps the most controversial aspect of Waxman's first bill is a provision that would allow more than one company to share marketing exclusivity if simultaneous development could be demonstrated. Abbey Meyers, executive director of the National Organization for Rare Disorders (NORD), originally opposed any form of 'shared exclusivity' but now supports the bill. She regrets the need for changes but recognizes that "many in Congress are very disturbed by what they perceive as abuses of the act". Genetics Institute, whose version of EPO is blocked by the orphan drug status of Amgen's EPO product (see *Nature* 344, 800; 1990), left the Industrial Biotechnology Association (IBA) in March after the trade group voted against changes to the Orphan Drug Act. Gabriel Schmergel, president and chief executive officer of Genetics Institute, praises Waxman's initiative as a "creative way of fine tuning [the act] and still retaining the incentives for the true orphans".

According to Schmergel, EPO, the biggest drug to come out of biotechnology, has worldwide sales estimated at

about \$450 million for 1990. "In the second year of its history, EPO is going to outsell, by a factor of maybe two, tissue plasminogen activator, and its most important indications are not yet approved", says Schmergel. "Commonsense tells you it's not an orphan." But Amgen's Mark Brand argues that, without the protection of orphan drug status, "we wonder whether or not there will be sufficient private-sector investment".

The proposed amendments would also require the FDA to assess future population growth before making orphan drug designations, which could have important implications for AIDS drugs. Moreover, drugs would lose their exclusivity if the

AIDS TESTING

Postal tests on the way

Washington

ENCOURAGED by the success of the drug AZT in treating people infected by the HIV-1 virus but showing no symptoms of AIDS, the US Food and Drug Administration (FDA) has lifted its opposition to AIDS testing outside clinics and hospitals, a decision that could lead to the marketing of home test kits for HIV-1 infection in the United States. Home testing had previously been ruled out because counselling and treatment for those testing positive could not be guaranteed.

FDA officials have accepted an application from University Hospital Laboratories Corporation (UHLC) for a licence to market a home blood-collection kit, which would allow users to mail blood samples to a testing laboratory and telephone for the results. On receipt of test results, expert counselling would be provided by telephone.

To avoid mailing potentially hazardous liquid blood samples, buyers of the UHLC kit would impregnate a small piece of filter paper with blood from a pin-pricked finger. The company's clinical trials show that the method is as reliable as tests done on liquid samples drawn by syringe. UHLC would then perform the standard HIV-1 antibody test, with the follow-up Western blot test recommended by the FDA to exclude false positives.

Two years ago, the FDA refused to review similar applications because of fears about the reliability of home test kits and the effectiveness of telephone counselling. But in an abrupt policy shift, the FDA recently wrote to more than 20 biotechnology companies saying it would now be prepared to look at applications.

UHLC's demonstration that an HIV-1 antibody test can work satisfactorily on blood samples collected at home was a factor in the change of policy, according to

treatment population exceeded the 200,000 limit. The second bill would extend the tax-credit programme to include human clinical trials and animal testing.

The issue has divided not only the biotechnology and pharmaceutical industries but also the trade associations.

Pamela Bridgen, executive director of the Association of Biotechnology Companies, sees Waxman's proposals as a "positive step". Both the IBA and the Pharmaceutical Manufacturers Association, however, oppose changes that would undermine what they believe to be the Act's key incentive — market exclusivity. There is likely to be heavy lobbying in government circles as both sides rally for support. As Schmergel says, "it's going to be a fight."

Diane Gershon

Paul Parkman, director of the FDA's centre for biologics. But a more important consideration was that AZT has recently been shown to be effective in treating asymptomatic carriers of HIV-1 as well as patients with full-blown AIDS. Parkman says that the FDA has softened its stance on home testing because anybody testing positive can usefully be treated with AZT, a benefit that federal officials believe outweighs the risk associated with telephone counselling.

But AIDS organizations oppose the FDA's policy shift. They still consider face-to-face counselling and the availability of back-up clinical support to be essential components of any test protocol. Jean McGuire, executive director of the US AIDS Action Council, describes the FDA's move as "worrisome". The biggest challenge posed by home testing is not the threat of inaccurate results or a breach of confidentiality but the problem of counselling, she says.

Robert Thewes of the National Association of People with AIDS doubts whether the home testing scheme could work without endangering the anonymity of its users, a problem heightened, he says, by the fact that anyone testing positive for HIV-1 in the United States runs the risk of losing health care insurance and possibly his or her job.

Nevertheless, UHLC spokesman Elliot Millenson believes that more Americans would willingly be tested for HIV-1 infection if they could do so in the privacy of their own homes, and that the advantages of home test kits for gathering epidemiological data would be considerable. In a survey conducted by UHLC, about 60 per cent of respondents said they would prefer a home test to a conventional test in a doctor's surgery or a clinic.

David Concar

EMBRYO RESEARCH

Pro-life actions backfire

London

LAST week's support for human embryo research in Britain by an overwhelming 364 votes to 193 in the House of Commons (see *Nature* 344, 799; 26 April 1990) came as something of a surprise. Most pro-research lobbyists had expected victory, but both they and their 'pro-life' opponents had been predicting a close result in the run-up to the debate (see *Nature* 344, 691; 19 April 1990). In a second vote, Members of Parliament (MPs) decided by 362 votes to 189 to set up a statutory licensing authority to regulate research on embryos that are 14 days old or less.

Professor Robert Winston, president of PROGRESS, the leading pro-research lobbying group, describes the majorities as "a vote of confidence in the integrity of British science and medicine". He says he perceived a shift in opinion among MPs, in favour of continued research, in the week preceding the vote. The publication of his own group's research paper, describing an *in vitro* technique to determine the sex of fertilized embryos and thus to screen for sex-linked genetic diseases (*Nature* 344, 768; 19 April 1990) was a factor, he thinks: the paper illustrated the "genuine benefit" of research.

Winston says the anti-research lobby's campaign undermined its own cause. Frank Cook, a Labour MP who came to the debate with his vote undecided, believed both sides had made misleading

claims, but said "the confusion caused by the pro-lifers is the most obfuscating".

One significantly counterproductive act, according to Christine Lavery of the pro-research Genetic Interest Group, was the decision by the Society for the Protection of Unborn Children (SPUC) to send a life-sized model of a 20-week-old fetus to all MPs. The move was designed to influence the following day's votes on the upper time limit for abortion, but it backfired: MPs voted for a 24-week maximum rather than the 22 weeks or less which had been predicted. Lavery says the distasteful models also turned "wavering" MPs against SPUC's anti-research arguments. SPUC led the pro-life campaign on both issues.

During the embryo research debate, several MPs argued that accepting research would be the beginning of a "slippery slope". Conservative MP Alan Amos said scientists would soon call for extensions to the 14-day limit. But Winston rejects this claim: scientists and doctors take their moral responsibilities "extremely seriously" he says.

The large pro-research majority will be reflected in the composition of the committee that will now consider the Human Fertilization and Embryology Bill in detail. This should mean that its passage through parliament is not delayed by the addition of "nitpicking" amendments, Winston says.

Peter Aldhous

ABORTION

RU486 under attack

Paris

IN a report sent to the French press and signed by Professor Pierre de Vernejoule of the Necker Hospital, a group of doctors and medical researchers claims that there are serious medical and economic drawbacks to the abortifacient drug RU486, which is being increasingly used within the French health service. But the group, although it calls itself a "commission of inquiry", has no official status, and its conclusions are derived from published literature and statistics rather than from any original research. Roussel-Uclaf, the maker of RU486, has denounced the group's work as "an amazing piece of intellectual dishonesty".

According to the group's analysis of accounts of the 30,000 documented abortions so far carried out with the drug, heavy bleeding occurs in 90 per cent of cases, and some women have needed blood transfusions. This, together with a need to verify, possibly using ultrasound scanning, that the fetus has been expelled, requires a level of medical support which, the report says, would rarely be available

in developing countries, where hopes have been raised that RU486 offers an easy means of population control.

The cost of this support, in the group's opinion, also makes questionable the true utility of the drug as an alternative to surgery. Whereas surgery is 99 per cent efficient and the abortion is completed in seven hours, RU486 is 95 per cent efficient and requires at least three consultations, including an ultrasound scan.

But at Roussel Uclaf the report has been dismissed out of hand. According to Dr Catherine Dubois, responsible for promoting RU486 outside France, there have only been two cases of serious side effects in the 30,000 abortions carried out with the drug.

Dubois does not deny the drawbacks, admitting that RU486 has been found safe only for use in countries with "luxury medicine", such as France and Britain, and then only under strict conditions: the drug must be used with prostaglandins, less than 49 days after conception and with guarantees of medical supervision.

Peter Coles

SOLAR POWER

The sunny side of the street . . .

Munich

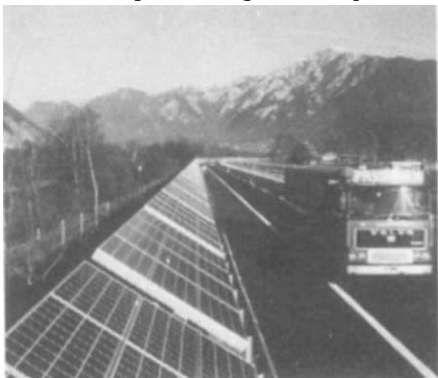
A Swiss entrepreneur has come up with a new solution to one of the major problems of using solar energy — where to put all the solar cells. Hundreds of acres are needed to generate any significant amount of energy, but meadows and mountainsides would be blighted if they were covered with huge, ugly solar arrays.

Thomas Nordmann of Chur has persuaded the Swiss government to try installing solar cells alongside an Alpine highway. The cells, mounted for 880 metres atop a sound baffle, provide 100 kilowatts of power to the local electricity grid.

The cells were installed in late 1989 as the first stage in a large-scale experiment in exploiting unused areas for solar energy generation. During the next phase, already under way, Nordmann's firm, TNC Consulting AG, is installing solar cells along a railway line in Ticino in southern Switzerland.

Nordmann will have completed four test sites by 1996, after which the Swiss government will decide if it wants to continue the project.

Solar cells installed only in the most advantageous areas, says Nordmann, could ultimately provide 11,000 households with up to 45 megawatts of power at



Road move: Part of the half-mile of solar panels alongside an Alpine highway.

peak demand times in winter. If all potential highway sites in Switzerland were used, solar cells could by the year 2000 provide 375 megawatts, as much as a medium-sized conventional power plant.

But the price will have to come down. At present, solar energy from Nordmann's cells costs one Swiss franc (about \$0.68) per kilowatt-hour, which is three to ten times higher than the market price of electricity. By 2000, Nordmann hopes that solar energy will be competitive with traditional power sources, at least at the daytime hours when demand is at its peak. The costs would come down through economies of scale and technical improvements, he says.

Nordmann has no patent on his idea. Others are free to copy it, he says, "but if it's going to be a poor copy, maybe they had better call us". Steven Dickman

But what is the problem?

Washington

If there was a consensus at last week's National Institutes of Health (NIH) conference on data ownership, it was that such meetings are a waste of time. One scientist after another declared that there was no serious problem with the way they now keep, share or record their data. "Why are we here?" one asked.

Why indeed? For most researchers, the *status quo* works well. Although federal regulations assign academic research grants to universities rather than to individual researchers, it is generally understood that principal investigators can do with their data what they will (patent issues excepted).

Most scientists say they have no problem getting data from other researchers on request, and the right of a scientist to be first to analyse and publish his or her data is widely respected.

But recent cases when the system has broken down (see accompanying story) have attracted public and Congressional attention, which has put pressure on the federal science agencies, especially NIH, to set matters straight.

Scientists have found fraud and misconduct becoming hot issues despite their professed belief that nothing is seriously wrong. The reasons for this outbreak of concern remain obscure: it may be, as a study from the NIH Office of Scientific Review suggests, that science is now big money, and the profit motive has become impossible to ignore. Or it could be increasing competition for funding. Collaboration of increasingly large numbers of scientists in research projects is another trend that can lead to disputes.

The main worry among those at the NIH meeting was that the government (in this case, the Public Health Service, PHS) would overreact to congressional pressure and impose draconian regulations on data management. At present, PHS requires recipients of grants to retain data for three years after the conclusion of the grant. The intention seems to be to preserve financial records in case of an audit, but the rules can be interpreted to include all scientific data, even laboratory notes or intermediate results.

PHS is now in the process of "clarifying" the rules. But with Congress breathing down its neck, it may choose to go for the broad interpretation. If that requires scientists to keep every scrap of paper and computer file generated by an experiment, data management could become burdensome indeed.

Nevertheless, the need to keep some data, even in a preliminary state, is generally accepted. There was little disagreement at the NIH meeting that it should be possible to double-check a scientist's

work. Who should double-check is a different question: universities say they do not want the responsibility, and few researchers want government intervention.

For lack of a better alternative, the scientific journals have become a favoured candidate. Drummond Rennie, deputy editor of the *Journal of the American Medical Association*, left the door open to the possibility. "Data should be retained so that journal editors might, if they wish, examine it", he said at the NIH meeting. But he was the only editor invited. Other journals (including this one) have resisted pressure to become data police. The peer-review process is already onerous enough, those journals say, and asking reviewers to inspect reams of raw data could overload the system to breaking point.

Data sharing is another battle in the making. Enormous computer-generated data sets are beginning to stretch storage and distribution resources. Several speakers at the NIH meeting said that "peer pressure" will remain a sufficient incentive to keep the data flowing between scientists, but in certain fields, such as gene sequencing, crystallography and epidemiology, access to data is already a problem.

Biomedical data in particular are of growing commercial value, and sharing them with other researchers carries risks.

Yet most of those at the NIH conference said they would rather work out the problems themselves than have regulations setting rules for data sharing. Non-binding "principles", determined by the professional societies or journals of each discipline, should set the norms of scientific behaviour, they said. But for how long will such measures keep the regulatory wolf at bay? **G. Christopher Anderson**

ICI's perishing plastic



LAST week chemicals company ICI launched what is claimed to be the first truly biodegradable plastic made from renewable resources. 'Biopol' is a form of the energy-storage molecule of the bacterium *Alcaligenes eutrophus*, poly(hydroxybutyrate-co-hydroxyvalerate), and has been chosen by Wella for one of its Sanara shampoos. Bottles, shaped like those shown above in an advanced stage of degradation, should appear in West German shops in May. □

Data case turns ugly

Washington

STILL lurking behind the debate on data ownership is the convoluted case of Erdem Cantekin, a University of Pittsburgh researcher whose public disagreement with his boss could cost him his job.

Four years ago, Cantekin was director of research for otolaryngology at the Children's Hospital. He had been hired by Charles Bluestone, director of the hospital's Otitis Media Research Center. But the two fell out when Cantekin disputed the results of clinical trial, for which Bluestone was principal investigator, of an antibiotic used to treat ear infections in children (*Nature* **340**, 668; 1989).

When Bluestone and Cantekin sent contradictory analyses of the data to the *New England Journal of Medicine*, editor Arnold Relman asked the university which was the "authorized" paper. The university said, in effect, that Bluestone owned the data and held sole right to publish. The university found Cantekin guilty of unethical behaviour for submitting an unauthorized analysis, stripped him of his duties and moved him to a remote office.

In August 1989 Cantekin appealed directly to the president of the university, but last month the president's five-person advisory panel found Cantekin guilty again. By submitting the dissenting manuscript without clearly marking it a dissent (although a cover letter did explain as much), Cantekin had committed a "serious violation of scientific and academic integrity", the panel decided.

The university informed Cantekin in a letter last week that it had begun the formal process of removing his tenure. Cantekin says he will ask the university president to follow due process and convene a formal hearing board on the issue. He is also considering initiating a lawsuit. One charge he expects to level is that the university last month moved him to an office above a grocery store six blocks from the hospital. Security guards video-taped his reaction to the move, Cantekin says.

University officials say that the move was necessary to permit renovations, and that other scientists will have to move too. The move was videotaped, they say, so that Cantekin could not claim that his belongings were damaged or stolen.

A congressional report due out next month from Representative Ted Weiss (Democrat, NY) will feature the case, among others, to illustrate the scope of the data-ownership dispute and scientific misconduct. The NIH Office of Scientific Integrity is also reviewing the case.

G. Christopher Anderson

AUSTRALIAN UNIVERSITIES

Just a Cambridge circus?

Sydney

In the first case of its kind, the Australian ombudsman is to investigate the Australian National University's appointment procedure, following accusations that a 'Cambridge cabal' unfairly controls the prehistory department at the university. It is alleged that University of Cambridge graduates of archaeology, and their students, have been given a monopoly of tenured positions in the department.

Johan Kamminga, previously visiting fellow in the department of prehistory within the Research School of Pacific Studies, has claimed that the cabal was established in the early 1960s, when the department was established.

According to a report presented by Kamminga to the ombudsman, in the years 1965-87 the proportions of UK students in the prehistory department were 25 per cent of the total intake. "These proportions are six times higher than the equivalent figures for the research school as a whole", Kamminga says.

Work at the school was reviewed in 1978 and then, according to Kamminga, the focus of research was switched, from Papua New Guinea and the Pacific region to Australia's trading partners — Japan and the South-East Asian countries. The school was encouraged to take graduate students from these areas.

But, says Kamminga, the department of prehistory has not done so, even though up to 40 per cent of the graduate students

Australian is equivalent to being a black South African. We're allowed to go for junior positions but British graduates get the more senior positions", Kamminga says. Brian Martin, lecturer in the sociology of science and technology at the University of Wollongong, supports Kamminga's claims, describing the department of prehistory as a "Cambridge academic colony down under". Professor Gerard Ward, director of the research school, disputes Kamminga's claims. "There is no preponderance of Cambridge graduates in the prehistory department. Some [Cambridge graduates] were appointed in the early sixties when there were no Australians at that level of seniority. The situation is now different,

HUMAN FRONTIER SCIENCE PROGRAM

Turned back at the door

Sydney & Tokyo

AUSTRALIA, Switzerland, and Sweden, the first non-summit nations to seek membership of Japan's Human Frontier Science Program (HFSP), will have to renegotiate the terms of their entry following a decision made last week by the seven summit nations (Canada, France, United States, Italy, West Germany, the United Kingdom and Japan) and the European Community at a HFSP board of trustees meeting in Strasbourg. But by then Australia may no longer have a budget for the programme.

Frontiers, a Strasbourg-based foundation that supports international research on the brain and molecular biology, was established by Japan in collaboration with the other summit nations. Japan supplies nearly all of the programme's current annual budget of \$15 to \$20 million (France and Italy also make small contributions). But all summit nations have an equal say in the running of Frontiers through their representatives on the programme's council of scientists and board of trustees, Frontier's highest governing body.

Switzerland, the first non-summit nation to seek membership, last year offered to contribute \$150,000 a year in return for one seat on the council of scientists (each summit nation has two seats). Australia and Sweden made similar offers. The Swiss proposal was discussed at the first meeting of the board of trustees last November. But despite enthusiasm from some summit nations, including Japan, representatives of other nations insisted that rules for non-summit membership should first be drawn up (*Nature*, 342, 601; 1989).

Provisional rules were established at last week's meeting but the board also decided to "re-negotiate" the offers made

we have PhD graduates coming from Australian universities." The one tenured position offered in the department of prehistory in the past ten years was awarded to a candidate with an undergraduate degree from Cambridge.

Professor Dennis Pearce, the Commonwealth ombudsman, says he will investigate the failure of the university to appoint Kamminga to two positions. "I will be concentrating on university appointment procedures. It would be presumptuous of me to second-guess academic appointments or to launch forth on an inquisition of the Cambridge connection", Pearce said. But the ombudsman also says that the allegations of cronyism are relevant to Kamminga's claim that he was unfairly treated. "It may be that we will end up investigating the Cambridge allegations", he said.

Tania Ewing

You should see the backs at
this time of the year: one mass
of daffodils...



in other departments in the school are from South-East Asia and Japan.

In the latest review, carried out last year, the university recommended that the department canvas more widely in Australia for postgraduate students. "It has been consistently the case, since the early sixties, that the department has fiddled appointments by stacking the selection committees with Cambridge graduates who, in turn, give appointments to other Cambridge graduates. Being

by the three non-summit countries, according to Toichi Sakata, director of the HFSP office of Japan's Science and Technology Agency. The decision to renegotiate places Australia in an awkward position.

Australia's International Science and Technology Advisory Council (HSTAC) has A\$285,000 to promote Australia's attempts to join the Frontier programme, according to Fiona Oliver of the Department for Industry, Technology and Commerce (DITAC). But the money has only been allocated until the end of this financial year (June 30) and ISTAC "is keen to get it out of the coffers by the end of May" in case it has to be returned to general government accounts, Oliver says. However, a decision on Australian membership cannot be made so quickly.

Under the rules drawn up last week, the council of scientists must first judge whether an applicant country is capable of contributing scientifically to the programme. The next council meeting, at which Australian, Swedish and Swiss membership can be discussed, will not be held until the end of May. Then it will be up to the board of trustees to decide an appropriate financial contribution from each country on a "case by case" basis, Sakata says.

If all goes smoothly it is Sakata's "personal hope" that a decision "one way or another" on the three countries can be made by the end of August when STA and the Ministry of International Trade and Industry will finalize their fiscal 1991 budget requests for Frontiers.

Australian government officials are not prepared to comment on the delay until they receive details of last week's meeting from their embassy in Tokyo.

Tania Ewing & David Swinbanks

Missing link at Sellafield

SIR—In 1927, when Hermann Muller showed that X-rays induce mutations in the common fruitfly *Drosophila melanogaster*, he did so by irradiating male fruitflies and looking for lethal mutations in their offspring. Within a year, most of the mutations were shown to be associated with deletions, inversions and translocations in the chromosomes. We now know that cells can repair such breaks. But in cells that divide repeatedly (such as the stem cells in the blood and skin and spermatogonia), the broken pieces sometimes rearrange themselves during mitotic division. Once chromosomes have been rearranged, the cell has no repair mechanism: if the rearrangement is passed down through the germ cells, it is in the genome for ever.

That mutations can be caused by breaks in chromosomes was shown by a combination of cytology and genetics in 1928; it was confirmed in the 1930s when the banding pattern of giant salivary chromosomes gave *Drosophila* cytologists increased resolution; it is the basis of much of the present-day search by molecular biologists for genes involved in human cancer. The breakpoints in chromosomal rearrangements associated with particular somatic (and hereditary) cancers are being used to find and characterize the genes they have disrupted.

Your leading article is correct in stating that statistics cannot prove causality

(*Nature* 344, 90; 1989). But basic science can. It is time for the scientific community to stand up and say so, with all the authority it can and should command. A spokeswoman for British Nuclear Fuels Limited is quoted as saying that the Gardner report into cases of leukaemia among children of workers at Sellafield "indicates a link but does not show a causal relationship" (*Brit. med. J.*, 300, 627; 1990). Indeed. But molecular genetics can do better than that.

Muller also showed that it is the cumulative dose of radiation that is important for heritable effects, not the dose received on a single occasion.

If I were a parent at Sellafield, I would not be too worried about face-to-face interviews. I would be asking for chromosome spreads and the very best that molecular genetics can offer. We should make sure that those families get help without a struggle. Childhood leukaemias will not be the only dominant mutations. And there are all the recessives.

GUIL WINCHESTER

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Japanese invention

SIR—A recent book review (*Nature* 343, 225–226; 1990), which discusses originality in Japanese research, will be welcomed by many Japanese scientists. However,

when it will reach our thirsty laboratories, but, since it usually takes some time from the formal announcement to the actual availability of the grants, they are not likely to be at the disposal of local groups for two or three months at best. The dates reported are for groups at Turin University, but they are more or less the same for the whole country. So, in five years, only four annual grants have been made, with a net saving of more than 20 per cent for the state budget. Additional handicaps are provided by constantly changing paperwork and application rules. No one seems to have protested against this rather bold way of managing public funds, and everyone is happy when the beloved money arrives.

The approach has survived several changes of ministers and even a change of ministry (last year, universities were separated from the Ministry of Education and a new Ministry for University and Scientific Research was created). It is so smooth and effective that other governments might consider following the Italian road.

DAVID LOVISOLO

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for this very reason, it is incorrect to cite the quick introduction of television broadcasts and the large share of Japanese-made television sets in the US market as a case of efficient imitation of Western science.

In fact, the TV monitor was invented and built by a Japanese scientist, Kenjiro Takayanagi, in 1926. Many prototype TVs had been built before his invention; however they were of a mechanical type, having no similarity with current TV monitors, which are based on the 'all-electronic' TV made by Takayanagi. He also invented the mechanisms of a TV camera in 1930 independently of Dr V. Zworykin of RCA in the United States, even though he could not build it due to the weak technological background in Japan at that time. Thus Takayanagi should be credited as the inventor of TV monitor, and perhaps as the last and the only living inventor in the tradition of Thomas Edison.

The reason for the 14-year delay (described in the review) in TV broadcasting in Japan was the intervention of the Second World War. The first test broadcast in Japan occurred in 1939, as in the United States. But in Japan, all TV research and broadcast attempts were suspended by the Japanese government in 1941–42, and were subsequently restricted by the United States until 1950.

The imbalance between the advancement of technology and inferiority in basic research in Japan seems to be mostly due to the uneven allocation of brilliant people in Japanese job markets. Most excellent students in Japan get a job in a private company as soon as they have obtained their bachelor's degree. However, masters' and PhD graduates are routinely rejected, or at most treated equivalently with bachelor's degree by many companies. Even though some changes have been made, openings are mostly restricted to applied areas such as engineering. Thus the imbalance in technology and science is strongly backed up by the human factor as a cultural consequence of Japanese society.

Japanese TV technology should be cited as an excellent example of the originality of Japanese invention, not as a case of imitation. The Japanese should not be viewed as excellent imitators lacking originality. It is Japanese culture that suppresses the development of originality in basic research. The apparent lack of originality in Japan's basic science, together with an excellence in imitation, is mainly due to the different 'allocation of originality' in Japan, as in the case of TV invention, which is in an applied field.

JIM YOSHIMURA

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Italian knowhow

SIR—Although the issue of cuts in money given to universities by governments has been bothering scientists — and others — in many countries, and your journal has provided accurate and often critical coverage, little attention has been paid to the Italian way of problem-solving in this field, which may prove the ultimate superiority of Mediterranean pragmatism.

Government funds for research are on the one hand managed locally by the universities, and on the other given directly by the ministry to research groups — or, better, to consortia of research teams. The latter amount to 40 per cent of the total of funds given each year (and are commonly referred to as "40 per cent funds").

The amount of these funds has dropped in recent years in real terms, but this is not the real trick. Our government has chosen a more subtle and more enterprising way, by stretching the duration of the financial year. Funds for the year 1985 were actually available to the research groups in May 1985; 1986 funds in October 1986; 1987 funds in January 1988. Things went better the following year, as 1988 funds arrived in January 1989; as for the 1990 money, no one knows, at the moment,

The origins of environmentalism

Richard Grove

There's nothing new in worries about man's effect on the natural world. Indeed, that history runs deeper (and wider) than latter-day environmentalists would have it.

ENVIRONMENTAL matters are now a central part of the political agenda in almost every country. Ideas about conservation and sustainable development have become highly politicized, and the prescriptions of environmentalists are receiving popular support of a kind that was previously heard only from a minority. But although the level of public interest in global environmental degradation is something new, the history of anxiety over the environment is not; on the contrary, the origins and early history of contemporary Western environmental concern and attempts at conservation lie far back in time¹.

Early worries over the effects of Western economic forces, on tropical environments in particular, emerged as a corollary of, and in some sense as a contradiction to, the colonization of the world by Europeans. Most attempts to understand the roots of conservationist responses to the destructive effects of man on nature have been largely confined to Europe and North America. Early environmentalism has generally been interpreted as a local response to the conditions of Western industrialization, while conservation has been seen as deriving from a specifically North American setting — Anglo-Americans such as George Perkins Marsh, Henry David Thoreau and Theodore Roosevelt are, for most people, secure in the pantheon of conservationist prophets². As a result the genuine antecedents of contemporary conservationist attitudes and policies have been overlooked. In particular, and largely for quite understandable ideological reasons, little account has been taken of the significance of the colonial experience in the formation of Western environmental attitudes and critiques (see, for example, ref. 3). Instead, it has simply been assumed that attempts to respond to environmental change in the tropics derived exclusively from metropolitan and northern attitudes.

In fact the converse was true. As colonial expansion proceeded, the environmental experiences of Europeans and

indigenous peoples living at the colonial periphery played a steadily more dynamic part in new European evaluations of nature and in the growing awareness of the destructive impact of European economic activity on the newly 'discovered' and colonized lands. After the fifteenth century, the global framework of trade

ized. For example, the notion that the Garden and Rivers of Eden might be discovered somewhere in the East was a very ancient one in European thought⁴. The European expansion during the Renaissance offered the opportunity for this quest for Eden to be realized alongside the other more obviously economic projects of early colonialism.

During the fifteenth century, the task of locating Eden and re-evaluating nature had already begun to be served by the appropriation of the newly discovered tropical islands as 'Paradises'. Dante's siting of an 'Earthly Paradise' in a 'southern ocean' is a case in point. This role was reinforced by the establishment of the earliest colonial botanical gardens on islands and on one mainland 'Eden', the Cape of Good Hope. Such projections were not easily confined, and they soon expanded beyond the botanic garden to encompass large tropical islands. Subsequently, India, Africa and the Americas, with their large 'wild' landscapes apparently little altered by man and their huge variety of plants (no longer confinable, as they had been, to one botanic garden), became vulnerable to colonization by an ever-expanding and imaginative symbolism. Frequently, such notions were closely allied to a stereotyping of luckless indigenous popula-

tions as 'noble savages'.

Paradoxically, the full flowering of Edenic thinking during the mid-seventeenth century coincided with the realization of the threats posed by the economic demands of colonial rule on previously uninhabited oceanic islands. There are descriptions of the damaging effects of deforestation and European plantation agriculture on the Canary islands and Madeira (the "wooded isle") after about 1300 and in the West Indies after 1560. In the Canary islands, complex irrigation systems formed part of an early technical response to the aridity that followed on despoliation. In the West Indies, particularly on Barbados and Jamaica, several attempts were made to try to prevent excessive soil erosion in the wake



Paradise lost? Mauritius in 1835, showing (white) the extent of deforestation: the island had been covered by trees 150 years before.

and travel provided the conditions for a process by which indigenous European notions about 'nature' were gradually transformed by a plethora of information, impressions and inspiration from the wider world. In this way, the commercial purposes of European expansion produced a situation in which the tropical environment was increasingly used as the symbolic location for the idealized landscapes and aspirations of the Western imagination. Shakespeare's play *The Tempest* and Andrew Marvell's poem *Bermoothes* stand as early examples of this cultural trend.

These aspirations then became global in their reach and increasingly exerted an influence on the way in which newly appointed lands and peoples were organ-

of clearance for plantations⁵.

Some of the worst consequences of early colonial deforestation occurred on St Helena and Mauritius. It was on these islands that a coherent and wide-ranging assessment of environmental degradation first emerged. Anxieties about soil erosion and deforestation had been expressed in much earlier periods, and are to be found in the literature of classical Greece, imperial Rome and Mauryan India, and in a sporadic fashion in the annals of the early Spanish and Portuguese colonial empires. But it was not until the mid-seventeenth century that awareness of the ecological price of capitalism started to grow into a fully fledged theory about the limits of the natural resources of the Earth and the need for conservation.

Sensitivity

Ironically, this new sensitivity developed as a product of the specific, and ecologically destructive, conditions of the commercial expansion of the Dutch and English East India Companies and, a little later, of the *Compagnie des Indes*. The conservationist ideology to which it gave rise was based both upon a new kind of evaluation of tropical nature and upon the highly empirical and geographically circumscribed observations of environmental processes that tropical islands made possible.

Some historians have argued that the colonial experience was not only highly destructive in environmental terms but that its very destructiveness stemmed from 'imperialist' attitudes towards the environment⁶. On the face of it, this seems a reasonable idea, particularly when viewed in the light of events in southern Africa in the late nineteenth century, when a highly exploitative agricultural and hunting ethos at first prevailed. But on closer inspection the hypothesis of 'environmental imperialism' does not stand up at all well. Many colonial states were peculiarly open, during the first half of the nineteenth century, to the often radical agendas of the contemporary scientific lobby. Paradoxically, the colonial state in its pioneering conservationist role provided a forum for controls on the unhindered operations of capital for short-term gain which, it might be argued, brought about a contradiction to what is normally supposed to have made up the common currency of imperial expansion. Ultimately, the long-term security of the state, which any ecological crisis threatened to undermine, counted for far more than the interests of private capital bent on destruction of the environment. Indeed, the absolutist nature of colonial rule made possible forms of land management that, at the time, would have been very difficult to impose in Europe⁷. Colonial expansion also promoted the rapid diffusion of new scientific ideas between

colonies, and between metropole and colony, over a large area of the world.

The continuity of the kind of assessment of the ecological effects of colonial 'development' which had arrived by the early eighteenth century was dependent on a coterie of committed professional scientists and environmental commentators. These men, almost all of whom were medical surgeons and custodians of the early botanic gardens, were already part of the administrative machinery of the new trading companies. As company investment in trade expanded into an investment in territorial acquisition, the members of the medical and botanical branches grew steadily in number. In India, for example, in 1838, there were over 800 surgeons employed at one time in different parts of the East India Company possessions⁸. As time passed, increasing demands were made upon these highly educated and often independent-minded employees. During the early eighteenth century the need to understand unfamiliar floras, faunas and geologies, both for commercial purposes and to counter environmental and health risks, propelled many erstwhile physicians and surgeons into consulting positions and employment with the trading companies as fully fledged professional and state scientists long before such a phenomenon existed in Europe. By the end of the eighteenth century their new environmental theories, along with an ever-growing flood of information about the natural history and ethnology of the colonies, quickly diffused through the meetings and publications of a whole set of 'academies' and scientific societies throughout the colonial world.

Again, the first of these societies appeared in the island colonies, particularly on Mauritius. This was no accident. In many respects, the isolated oceanic islands stimulated a detached self-consciousness and a critical view of European origins and behaviour, of the kind dramatically prefigured by Daniel Defoe in *Robinson Crusoe*. Such islands became, in practical as well as mental terms, an allegory of a whole world, and observations of their ecological demise were easily converted into premonitions of environmental destruction on a wider scale.

So, alongside the emergence of professional natural science, the importance of the island as a mental symbol was an essential stimulant to the formulation of ideas about environmental protection, as well as of ethnological identity. Half a century before the Falkland and Galapagos Islands provided Charles Darwin with the data he required to construct a theory of evolution, the isolated and peculiar floras of St Helena and Mauritius had already sown the seeds for a concept of rarity and a fear of extinctions that, by the 1770s, was already in the minds of French and British colonial

botanists. The scientific odysseys of Bougainville and Cook served to reinforce the significance of tropical islands.

So it is no surprise that it was on one of them, Mauritius, that the early environmental debate came to a head. Between 1768 and 1810, the island was the location for some of the earliest experiments in systematic forest conservation, pollution control and fisheries protection. These initiatives were carried out by scientists who, characteristically, were both followers of Jean-Jacques Rousseau and adherents of the kind of rigorous empiricism associated with mid-eighteenth century French Enlightenment botany. Their conservation measures stemmed from an awareness of the potentially global impact of modern economic activity, from a fear of the climatic consequences of deforestation and, not least, from concern over species extinctions. As a consequence the 'Romantic' scientists of Mauritius, and above all Pierre Poivre, Philibert Commerçon and Bernardin de St Pierre can, from hindsight, be seen as the pioneers of modern environmentalism. All of them held that responsible stewardship of the environment was a moral and aesthetic priority, as well as a matter of economic necessity.

After the annexation of Mauritius by the British in 1810, these environmental prescriptions were transferred to St Helena and eventually to India itself. From 1820, they were strongly reinforced by the writings of Alexander von Humboldt. Pierre Poivre, on Mauritius, had already been persuaded of the value of tree-planting and protection by his observations of Indian and Chinese forestry and horticultural methods, and his knowledge of Dutch botanic gardening techniques derived, circuitously, from the Mughal emperors. Humboldt's environmental writings, however, were guided by Indian thinking in a far more profound way. Much influenced by the orientalist writings of Johann Herder as well as by those of his own brother, Wilhelm, Alexander von Humboldt strove, in successive books, to promulgate a new ecological concept of the relations between man and the natural world which was drawn almost entirely from the holist and unitary thinking of Hindu philosophers. His subordination of man to other forces in the cosmos formed the basis for a wide-ranging and scientifically reasoned interpretation of the ecological threat posed by the unrestrained activities of man.

Scotsmen

This interpretation became especially influential among the Scottish scientists employed by the East India Company. These men, mainly medical surgeons trained in the French-derived Enlightenment traditions of Edinburgh, Glasgow and Aberdeen universities, were especial-

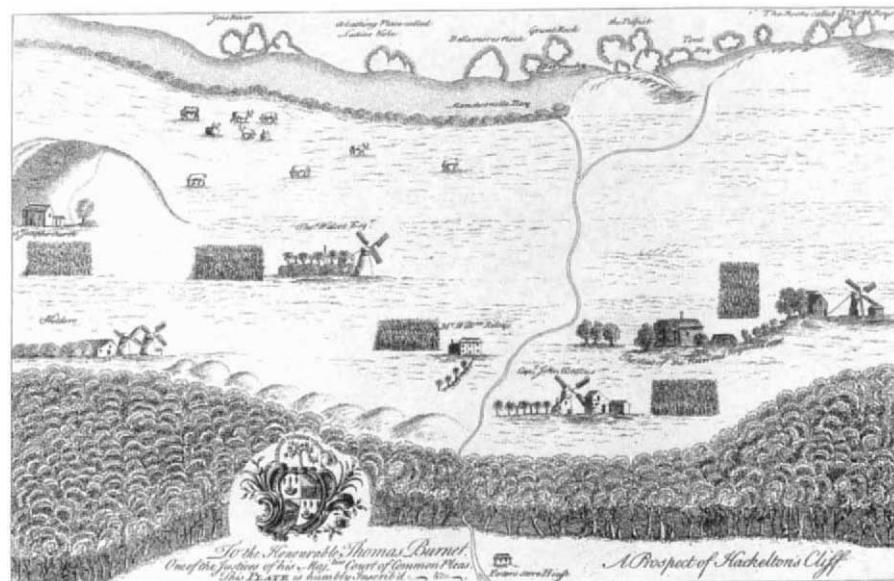
ly receptive to thinking that integrated the phenomena of deforestation, water supply, famine, climate and disease. Several of them, in particular Alexander Gibson, Edward Balfour and Hugh Cleghorn, became enthusiastic proselytizers of a conservationist message which proved both alarming to the East India Company and effective in providing the basis for the pioneering of a forest conservancy system in India⁹. For example, in 1847 the directors of the East India Company indicated their conversion to the need for conservation with a remarkable circular on the dangers of artificially induced climate change. The statement was also a request for information. The subject, they said

is one having strong practical bearing on the welfare of mankind, and we are anxious to obtain extensive and accurate information in regard to it. We desire, therefore, that you will furnish us with any that you may possess, and that you will institute enquiries in such quarters as may be likely to lead to the acquisition of particular facts bearing upon the question [ref. 10].

Summary

The request was taken up with alacrity by the Company Medical Service, and the results were summarized in 1852 in the "Report of a Committee Appointed by the British Association to Consider the Probable Effects in an Economic and Physical Point of View of the Destruction of Tropical Forests". This warned that a failure to set up an effective forest protection system would result in ecological and social disaster. Its authors were able to point to the massive deforestation and soil erosion which had occurred on the Malabar coast, with the resulting silting-up of harbours, as early evidence of what might happen in the absence of a state conservation programme. The report drew on evidence from around the world, and did not confine its analysis to India. Later, the forest conservation system set up in India, itself based in part on experience in Mauritius provided the model for most of the systems of colonial state conservation in South-East Asia, Australasia and Africa and, much later, in North America. (It should be noted, however, that the first warnings of the dangers of deforestation in India that were communicated to the colonial authorities had originated from indigenous Indian rulers as much as from the early colonial scientists. Moreover, some of the first large-scale attempts at re-afforestation in India were made by the Amirs of Sind between 1785 and 1840¹¹).

If there is a single historical lesson to be drawn from the early history of conservation under the East India Company and the *ancien régime* of Mauritius, it is that states can be persuaded to act to prevent environmental degradation only when their economic interests are shown to be directly threatened. Time and again, from the mid-eighteenth century onwards, sci-



Engraving from *The Natural History of Barbados* (1750), showing land given over to agriculture after forest clearance. The accompanying text speaks of soil erosion and the resulting land slips on the coast.

entists discovered that the threat of artificially induced climatic change, with all it implied, was one of the few really effective instruments that could be employed in persuading governments of the seriousness of environmental change. The argument that rapid deforestation might cause rainfall decline, decline in runoff and, eventually, famine, was one that was quickly grasped by the East India Company, fearful as it always was of agrarian economic failure and social unrest. Unfortunately, it often required an initial famine to lend credibility to scientists in the eyes of government and to provide the impetus for the state to intervene with environmental protection measures. In India, for example, serious droughts in 1835–39, the early 1860s and 1877–78 were all rapidly followed by the renewal of state programmes designed to strengthen forest protection, often with the aim of preventing droughts. Such legislation had the convenient by-product of increasing state control over land and timber supplies (often at the expense of common rights), but the central motive was an underlying fear of climatic change.

Likewise, the early pioneer of state conservation in the Cape Colony, John Crombie Brown, was able to secure government agreement to new forest conservation and grassland-burning prevention measures only after the disastrous drought of 1861–63 had wrought havoc on settler agriculture throughout the colony. In fact, that drought encouraged the development of a whole school of 'desiccationist' theory, closely related to a contemporary Indian counterpart, central to which was the conviction that most of the semi-arid tropics was undergoing long-term aridification, in part because of deforestation¹².

Theories of widespread climatic change acquired further credibility when a paper

was read at the Royal Geographical Society in London in March 1865 on "The Progressing Desiccation of the Basin of the Orange River in Southern Africa" by James Fox Wilson, a naturalist and traveller. He believed that the Orange River was "gradually becoming deprived of moisture", that "the Kalahari desert was gaining in extent" and that the desiccation was due to "the reckless burning of timber and the burning of pasture over many generations by natives"¹³. David Livingstone, present at the lecture, disagreed strongly with Wilson's analysis. Rainfall decline, Livingstone asserted, was a natural phenomenon due to geophysical phenomena. Another speaker, a Mr Galton, believed that the introduction of cheap axes into Africa by Europeans had promoted excessive deforestation and consequent drought.

Yet another member of Wilson's audience, Colonel George Balfour, of the Indian Army, struck a more caustic note. Rainfall decline in India, he believed, was caused principally by the deforestation activities of the whole community, including European plantation owners. Countermeasures were necessary. He had been informed that morning, he said, that in the West Indies the Government of Trinidad had passed a law prohibiting the cutting down of trees near the capital, in order to ensure a supply of rain. Balfour was quick to point out on this, as on other occasions, that in pre-colonial times it had been the practice of Indians to sink wells and "plant tops of trees" to encourage water retention. In another Royal Geographical Society debate, in 1866, Balfour pointed out, too, that "in the Mauritius the Government had passed laws to prevent the cutting down of trees, and the result has been to secure an abundant supply of rainfall".

The question of climatic change had

thus become international in scope by the mid-1860s. It was reinforced by more detailed research that raised the possibility that the very constitution of the atmosphere might be changing. Such views, the origins of the current 'greenhouse' debate, had found an early supporter in J. Spotswood Wilson, who presented a paper in 1858 to the British Association for the Advancement of Science on "The General and Gradual Desiccation of the Earth and Atmosphere"¹⁴. Wilson stated that upheaval of the land, "destruction of forests and waste by irrigation" were not sufficient to explain the available facts on climate change, and that the cause lay in the changing proportions of oxygen and carbonic acid in the atmosphere. Their respective ratios, he believed, were connected with the relative rates of their production and absorption by the "animal and vegetable kingdom". The author of this precocious paper concluded with a dismal set of remarks. Changes in "the atmosphere and water" were

in the usual course of geological changes, slowly approaching a state in which it will be impossible for man to continue as an inhabitant . . . as inferior races preceded man and enjoyed existence before the earth had arrived at a state suitable to his constitution, it is more probable that others will succeed him when the conditions necessary for his existence have passed away.

The raising, as early as 1858, of the spectre of human extinction as a consequence of climatic change, was clearly a shocking psychological development. But it was consistent with fears that had been growing among the scientific community. Awareness of species rarity and the possibility of extinction had existed since the mid-seventeenth century as Western biological knowledge started to embrace the whole tropical world. The extinction of the auroch in 1627 in Poland and the dodo by 1670 in Mauritius had attracted considerable attention. Again, the mechanisms of extinctions, and the contribution of man to the process, first began to be clearly grasped on the tropical island colonies.

St Helena

On St Helena, attempts were made as early as 1713 to protect the St Helena redwood in the knowledge of its apparently imminent demise. Eighty years later William Burchell, the government botanist of St Helena from 1805 to 1810, and one of the first scientists employed by the East India Company, had acquired a systematic knowledge of the endemic plants of the island and had explicitly recognized the probability of species extinctions¹⁵. In part this recognition was based on Burchell's exceptional knowledge of world plant species. But he was also keenly aware of the rapid and continuing deforestation that was taking place on the island, despite attempts made to legislate against it as early as 1709. A knowledge of the

threatened and endemic character of the St Helena flora can also be found in the writings of William Roxburgh, the renowned superintendent of the Calcutta Botanic Garden, and Charles Darwin himself.

The publication in 1834 of Charles Lyell's *Principles of Geology*, a book which questioned the fixity of species and laid the basis for the modern understanding of geological change, gave a firm footing to the confused awareness of the phenomenon of extinctions that had already developed earlier among East India Company scientists such as Burchell on St Helena and Roxburgh in Calcutta. The question mark Lyell placed over *Genesis*-based concepts of geological processes stimulated a revolution in notions about the speed of environmental processes and, paradoxically, emphasized the apparent helplessness of man in the face of environmental change. By the early 1840s Ernst Dieffenbach, in his work on the fauna of New Zealand and the Chatham Islands, and then in his studies of Mauritius, had become more acutely aware of the potential for further rapid extinctions as European economic activity spread¹⁶. In Britain, Strickland had first been made aware of the extinction problem by his work on the palaeontology of the dodo and other extinct birds of the Mascarenes, and actually suggested that the entire colony of New Zealand should be made a nature reserve to save its remaining fauna¹⁷. This was at a time when the value of rare island faunas was being recognized in the formulation of theories about species origins, not only by Darwin but also by Strickland, Hooker and Dieffenbach¹⁸.

The appearance in 1859 of *The Origin of Species*, with its emphasis on the place of extinction in the dynamics of natural selection, served only to sharpen the dilemma of colonial scientists, many of whom were already aware of the part played by man in hastening species extinctions. This awareness, crystallized by Darwin's work, underlay many of the attempts made by early environmentalists to secure state controls over tropical deforestation. Hugh Cleghorn, for example, the first Inspector-General of the Madras Forest Department (set up in 1856) stated explicitly in several publications that uncontrolled deforestation would both cause the loss of potentially valuable species and prevent botanists from assembling the evidence for the evolution of species. But he seems to have considered that such arguments might not weigh convincingly with government, and chose to stress instead the more obvious economic hazards of climatic change and resource depletion.

The Origin of Species, however, went some way to making species protection a more valid concept in the eyes of government, and the period 1860–70 produced a

flurry of attempts to legislate for the protection of threatened species. Once more, the innovating context was an island colony, Tasmania, where a comprehensive body of laws, designed mainly to protect the indigenous birds, was introduced in 1860 after vigorous lobbying by J. Morton Allport, an amateur naturalist¹⁹. Other territories followed suit. For example, the colonial legislatures of Natal and Victoria (Australia) had both introduced laws to protect selected animal and bird species by 1865. Somewhat belatedly, Britain followed suit, introducing its first bird protection legislation in 1868. The timing of such species protection measures, all closely connected with the climate of opinion stimulated by Lyell and Darwin, offered a symbolic as well as practical opportunity to try to reassert control over a process of environmental degradation that was now understood to be worldwide in scale.

Survival

So, by the early 1860s, anxieties about artificially induced climatic change and species extinctions had reached a climax. Western-style economic development, spread initially through colonial expansion, was increasingly seen by more perceptive scientists as threatening the survival of man himself. The subsequent evolution of the awareness of a global environmental threat has, to date, consisted almost entirely in a reiteration of a set of ideas that had reached full maturity over a century ago. The pity is that it has taken so long for them to be taken seriously. □

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Cooperating molecules in biology

Cooperative phenomena, best known to physics, have an important function in biology that was first and most radically defined in an article published a quarter of a century ago.

COOPERATIVE phenomena are ill-defined, nowhere more so than in biology. The general idea is that the effect of the simultaneous action of separate entities may be greater than the sum of their separate effects. Order is one consequence. Physicists know cooperative phenomena best in phase transitions. In a ferromagnetic material below the Curie temperature, for example, the magnetization at a particular lattice site will be the more certainly identical with that of its immediate neighbours if there is such a degree of long-range order that other than immediate neighbours exert a similar effect. Order, and also disorder, are properties of the whole and not of the parts separately.

Usage among biologists is more casual. At one extreme, the word cooperative is wrongly used to describe circumstances when two molecules are necessary for the interaction of a third. Thus molecules of both an enzyme and a coenzyme may be needed to effect the transformation of a substrate molecule. Literally, the effect of the enzyme and coenzyme jointly is greater than the sum of their separate effects (in each case zero). But the reaction rate is determined simply by the product of the concentration of the two components, as the law of mass action would suggest. Happenings at one place do not help to determine what happens at another.

The classic case of cooperativity among the molecules of biology is that of the absorption of oxygen by haemoglobin, still something of a mystery despite the endless investigations since the discovery of the Bohr effect at the turn of the century. (The variation with pH of the affinity of haemoglobin for oxygen explains why haematologists are fond of measuring bicarbonate concentration in blood.) What does simpleton mass action say? The product of the haemoglobin and oxygen concentrations (with the partial pressure as a standard proxy) is equal to the concentration of the oxygenated form multiplied by an equilibrium constant.

So how does the proportion of oxygenated haemoglobin vary as the partial pressure of oxygen increases? Simple algebra says that the proportion of oxygenated haemoglobin will be $P/(K + P)$, where P is the pressure and K the equilibrium constant, which is known as the Michaelis–Henri equation. This gives a simple and plausible graph, increasing from zero at zero pressure to some plateau on which all

the haemoglobin in oxygenated. The exact shape of the curve is determined only by the equilibrium constant. The chief interest of the Michaelis–Henri equation is that it hardly ever corresponds with the truth.

What happens instead is a consequence of cooperativity, for which purpose it is crucial that haemoglobin in the blood is not a single protein molecule, but a tetramer. For those not already familiar with the argument, the standard textbooks provide a better explanation than the telegraphese that follows, which has a different purpose—partly that of celebrating how it proved possible to figure out what really happens without much help from a knowledge of the detailed structure of haemoglobin. (The very best way to hear the tale is to seize the next opportunity to listen to Dr Max Perutz on the subject.)

Doubters should read the classic paper by Jacques Monod, Jeffries Wyman and Jean-Pierre Changeux (*J. molec. Biol.* **12**, 88–118; 1965), published exactly a quarter of a century ago. The observations show that the proportion of oxygenated haemoglobin usually increases from zero with increasing partial pressure less quickly than simple-minded mass action would suggest, but that the upward slope of the curve then increases more quickly than would be expected. (The shape of the curve is physiologically beneficial in concentrating the absorptive capacity of haemoglobin in a relatively narrow range of partial pressure.)

The qualitative explanation is that the attachment of a first molecule of oxygen to one component of a tetramer eases the attachment of other oxygen molecules to the remaining components. So the phenomenon is strictly cooperative; the condition of one component of the tetramer conditions the others in the same sense. As textbook readers know, this in turn leads to the doctrine of allostery—the notion that proteins can be stimulated to change their shape and, in the process, their activity.

The neatness of the argument by Monod *et al.*, which reads like pure reason, is its generality. Allosteric proteins (24 were known then) are multimeric molecules, so why not assume that their components occupy equivalent positions, which says something about their geometrical symmetry. On this view, normal human adult haemoglobin, which has two

pairs of α globin chains and two β chains, is a dimer of components each with two absorption sites for oxygen.

The natural temptation would be to look for detailed mechanisms by which the attachment of a single oxygen molecule to one chain, with its characteristic haem iron atom, would influence the oxygen affinity of the others. Not for Monod *et al.*, who simply posit that allosteric change yields another tetramer in which the four units are again in equivalent positions. Moreover, although the function of the oscillation between one state and another may be crucially related to the attachment of oxygen molecules, the equilibrium between them must be definable without the intervention of oxygen. That makes one equilibrium constant. For haemoglobin, four others arise from the affinity for oxygen of the binding sites on the α and β chains in the two different allosteric states. Then it is simple calculation, as with the Michaelis–Henri equation, and simple demonstration that the more complicated relations that result do indeed correspond with reality.

All this is by way of introduction to what must be the most elaborate argument of this kind. The case is that of the oxygen-carrying haemocyanin with which evolution has endowed molluscs. The structure of the standard haemocyanin complex consists of ten linear protein units, each consisting of two protein monomers placed equivalently head to tail, which are arranged like the staves of a barrel to form a hollow drum. There may be 100 or more binding sites for molecular oxygen in each such hollow drum.

E. Di Cera of the Catholic University at Rome has now gleefully calculated the allosteric possibilities of these molecules (*Il Nuovo Cimento* **12D**, 61; 1990). It seems to be established that oxygen attachment to a binding site within a single barrel stave causes allosteric change in that stave alone, which can be catered for with the techniques of Monod *et al.*. Di Cera supposes that allosteric change in one stave of the barrel will influence its neighbours in its own image, and sets out to calculate the propagation of that influence around the barrel. He calculates the partition function and derives what thermodynamic properties he wishes. The model has the virtue that its parameters are physically defined as interaction energies. Observational data are well fitted. Who could ask for more? **John Maddox**

Leukaemia and radiation

H. John Evans

In February, Martin Gardner and colleagues¹ published the results of their study of the apparent excess cases of childhood leukaemia near the nuclear waste reprocessing plant at Sellafield, West Cumbria. Their suggestion is that the excess is due to the increased risk to children of fathers working at the plant. That conclusion, however, is questionable.

Gardner *et al.*'s report follows other investigations of leukaemia clusters near nuclear facilities^{2,3}. The authors analysed 52 cases of leukaemia, 22 cases of non-Hodgkin's lymphoma and 23 cases of Hodgkin's lymphoma in people under the age of 25 years born in West Cumbria and diagnosed between 1950 and 1985, and 1,001 controls matched for sex and age, and taken from the same birth registers as the cases. The authors focused on the 74 cases of leukaemia and non-Hodgkin's lymphoma, because there did not seem to be any association between Hodgkin's lymphoma and the variables studied that were thought to increase the risks. By cross-linking birth certificate entries to British Nuclear Fuels Ltd (BNFL) files on Sellafield, the authors discovered the occupations of the parents of 64 cases; ten of these cases had fathers who had worked at Sellafield. This compared with 383 local controls, of whom 54 had fathers who had worked at the plant.

Other industries

Most commentators, however, ignored the comparisons between the relative risks of leukaemia and non-Hodgkin's lymphoma for occupations at Sellafield and for those with other industries. Gardner *et al.* looked at the main industrial groups in West Cumbria that employed more than five per cent of the control fathers. They found that the relative risk of leukaemia and non-Hodgkin's lymphoma was roughly doubled for the children of Sellafield workers, but was also similarly raised for the children of farmers, iron and steelworkers, and chemical workers. There is no information about whether the chemicals in the environs of these workers are leukaemogenic, and no measure of the exposure, so that further analysis of these groups is not possible. But there are some findings that suggest an excess of childhood cancers among offspring of workers exposed to certain organic chemicals such as benzene, a known leukaemogen. For radiation workers, external radiation exposure is measured by a film badge, and it is this that allows a more detailed analysis of the Sellafield workers as opposed to those in the other industries.

Gardner *et al.* conclude that the excess of leukaemia and non-Hodgkin's lympho-

ma near Sellafield is not due to radionuclide contamination of the environment by nuclear waste discharges, or to eating local seafood or vegetables, or to playing on the beach. The authors found that 4 of the 10 case fathers, but only 3 of the 54 control fathers, had accumulated exposures of 100 millisieverts (mSv) or more before the conception of their child, and that this was associated with a six- to eightfold greater risk of leukaemia in the offspring. An association was also noted for those fathers who had received 10 mSv or more in the six months before conception.

The expected (or more than expected) increased risk of leukaemia with the age of the mother (relative risk ~4) or with previous diagnostic antenatal X-ray exposure (relative risk ~1.5) was observed, but could not account for the excess cases among the offspring of radiation workers. The authors say that their findings indicate an "effect of the radiation exposure on germ cells producing a mutation in sperm that may be leukaemogenic in subsequent offspring". Is there any evidence in support of this suggestion, and is it a realistic possibility?

Shu *et al.*⁴, studying childhood leukaemia in Shanghai, reported a roughly four times greater risk to children of fathers who had received ten or more doses of diagnostic X rays preconceptionally: the occupation of the fathers or whether they were receiving therapeutic drugs was not noted, and the findings are open to question because of recall bias. Similar increased risks were also found for the offspring of mothers employed in the chemical, metal refining and agricultural industries, and, in particular, in those associated with exposure to benzene and to pesticides. Also relevant is the absence of an excess of leukaemia in offspring of atom-bomb survivors at Hiroshima and Nagasaki (5 cases observed, 5.2 expected) in a population of 7,387 men who had been acutely exposed to a high dose (mean of 492 mSv)⁵. Additionally, Nomura⁶ demonstrated an approximate doubling of lymphocytic leukaemia in the offspring of mice exposed to high acute doses of X rays (360–5,040 mSv).

Gardner *et al.* refer to the Nomura studies as evidence for heritability of mutational change resulting in leukaemias in offspring of exposed parents, but argue that the absence of such effects in the offspring of survivors at Hiroshima and Nagasaki may be a consequence of their exposure to acute high doses in contrast to the low doses accumulated by the Sellafield workers. But Nomura used doses of the order of those received by the atom-

bomb survivors; his studies also revealed, as expected, an increased frequency of inherited congenital abnormalities among the offspring. It might be argued, therefore, that if doses of 100 mSv resulted in mutations of workers' sperm that lead to a six- to eightfold increase in leukaemia, then a considerable increase in a wide range of heritable diseases or anomalies among their children would also be expected. No such epidemic has been noted in the Sellafield area.

Inherited factors

Is there any evidence for inherited factors that predispose to human leukaemia and lymphoma? Aside from an association with the rare and well-characterized syndromes (for example, Bloom's, Fanconi's anaemia and so on) caused by single gene defects and associated with various physical abnormalities and defects in immune response, there is no unambiguous evidence that childhood leukaemia is associated with an inherited single gene. In those leukaemias in monozygotic twins that have been studied cytogenetically, each member of a pair shares the same abnormal marker chromosome, indicating a single transformation event and the transfer of leukaemia cells from one twin to the other by their shared blood circulation *in utero*. So twin studies are not helpful. And several consanguinity studies show that most leukaemias are not associated with an inherited predisposing recessive gene. There are, nevertheless, a number of well-documented cases of familial childhood leukaemia, but in most of these the possibility of a transmissible infectious agent or of common exposure to a leukaemogenic chemical cannot be ruled out.

Assuming, despite the lack of evidence, that one or more mutations acting within a given genetic background (multifactorially) could predispose to leukaemia, then are the exposures at Sellafield sufficient to produce such mutations and at the frequencies implied? Apart from data on acute X-ray-induced chromosomal mutations in human spermatozoa (which require a dose of ~4,000 mSv to double the background mutation frequency, and >20,000 mSv to raise it six to eight times)⁷ there is no information on viable inherited X-ray-induced mutations in man. But mouse studies show that the minimum X-ray dose to spermatozoa or spermatogonia required to double the spontaneous frequency of a wide range of different single gene defects is of the order of 1,500–3,000 mSv given as an acute exposure (see ref. 8): these doses are at least 15–20 times greater than the doses accumulated by the most heavily exposed Sellafield workers.

Although there is a lack of evidence for heritability of human leukaemias, they are nevertheless genetic diseases; but



Sellafield — do workers' offspring have a greater risk of cancers? The question remains open.

the genetic changes that characterize them are somatic genetic changes. Even though detailed pathological findings have not been reported, most of the West Cumbrian childhood leukaemias were acute lymphocytic leukaemias, the most common childhood leukaemia. About 50 per cent of acute lymphocytic leukaemias are characterized by the presence of one of several consistent chromosome translocations, which in a number of cases results in the activation of proto-oncogenes at the sites of chromosome exchange^{9,10}. These chromosome alterations, which are clearly causal factors in leukaemogenesis, are confined to the leukaemic cells and involve genes associated with growth, development, proliferation and differentiation. Such chromosome alterations would be inconsistent with the viability of an early embryo and are not found as constitutional changes in the normal cells of leukaemia patients. There is no information on chromosome changes for the Sellafield cases, but the highly unlikely inheritance of such changes would be associated with various other abnormalities as well as leukaemia.

Overall therefore, the evidence pointing to an inherited mutational change induced by low doses of radiation as a cause of the excess leukaemia among the offspring of radiation workers does not stand up to scrutiny. Are there other possible explanations apart from various nonspecific suggestions such as unusual genetic imprinting or induced suppression of the immune response resulting in an enhanced response to leukaemia-causing agents encountered postnatally? There are several. First, we cannot rule out the possibility of chance, for the main conclusion of the case control study rests heavily on four of the cases born to Sellafield fathers. Similar control studies are in progress for the nuclear plant at Dounreay in Scotland and at the atomic weapons

establishment and ordnance factory at Aldermaston (Berkshire) and Burghfield (Hampshire); the Dounreay results are soon to be published. Other studies are underway in France, Canada and the United States, but preliminary negative published data are so far insufficient to draw any conclusions. Perhaps the most important study is that proposed by the National Radiological Protection Board (NRPB) and the Oxford Childhood Cancer Research Group. The Oxford group has records on over 10,000 leukaemic children diagnosed in Britain since 1962, and the NRPB has a register of over 100,000 radiation workers with details of film-badge doses. Linking these two registers will be formidable but well worthwhile.

Chemical agents

A second possibility is that the film badge may not measure the relevant exposure to radiation (or, indeed, to other agents). It is unlikely that the most heavily exposed workers inadvertently carry radioactive dust home, but the possibility that these workers have internal gonadal contamination that could result in sperm being exposed to higher doses, or in radioactivity being transmitted to the female partner in seminal fluid, still exists. Indeed, the finding that the risk of prostatic cancer is increased among the most heavily radiation-exposed employees at Harwell and Aldermaston has led to the suggestion that some radionuclides are concentrated in the prostate¹¹. But radiation workers are exposed to more than radiation. For the four leukaemia cases with fathers in the highest radiation dose group, one of the fathers was an analytical chemist, one a fitter's mate and the others process workers. Whether these workers all worked in the same building and were exposed to a common chemical leukaemogenic agent is not known. The evidence

that preconceptual exposure to chemical agents induces leukaemia in animals, and probably also humans, is at least as strong as the evidence for radiation exposure, and there is no information on what chemicals are in the environs of the Sellafield workers. A possible carry-over of traces of leukaemogenic chemicals, for example, decontaminating chemicals, on the bodies and clothing of such workers cannot therefore be discounted, and neither can the possibility that the leukaemias are induced postnatally and not as a consequence of preconceptual exposure to a mutagen. If these diseases were indeed induced postnatally, and as a secondary consequence of radiation or chemical exposure, then man may be no different from chickens, mice, cats, cattle and gibbons, all of which suffer leukaemias as a consequence of viral infection. Studies on mouse strains harbouring a leukaemogenic virus show that activation of latent virus can occur after exposure of animals to radiation or other environmental insults. Human lymphoid cells in culture that harbour the Epstein-Barr virus can be made to release large quantities of replicating virus after exposure to DNA-damaging agents; whether a similar phenomenon is involved in clusters of childhood leukaemias is not known.

In view of the public concern about radiation and cancer, it is worth emphasizing that childhood leukaemia is a tragic but not a common disease. Its prevalence in the UK is about 1 in 2,000, and it is far less common than genetic abnormalities in the newborn that result in other forms of ill health and which are some 70 times more frequent. Although an excess of half a dozen childhood leukaemia cases has been noted over a 35-year period near Sellafield, over 12,000 cases occurred elsewhere in the UK over this interval. Gardner has said that his intriguing findings are "a pointer"; let us hope that they will point the way to a much needed understanding of the causal factors in this disease. □

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The mouse that wasn't immune

F. E. G. Cox

OVER the past few years it has become clear that humans can acquire some kind of immunity to schistosomiasis. Although the mechanisms involved are not clear, we do have a vast amount of information about immunity to schistosome infections in laboratory animals, of which the mouse is one of the most widely used. However, a series of recent reports¹⁻³ has pointed to some of the dangers inherent in attempting to draw too many conclusions from the schistosome-mouse model, adding fuel to the debate as to whether any of the existing laboratory models are realistic ones.

Schistosome infections begin when the aquatic larval stages of the parasite, the cercariae, penetrate the skin. The cercariae remain there for 4-5 days, acquiring a coating of host antigens before entering a blood vessel to be carried, as schistosomula, to the heart, lungs, gut and liver. During this passage, they gradually mature into egg-laying adults that normally reside in the blood vessels of the gut or bladder. *Schistosoma mansoni* is the best studied species and its hosts can be classified as permissive, in which the whole life cycle as far as egg-laying adults is completed, or non-permissive, in which the life cycle is curtailed *en route*. The rat is a non-permissive host whereas humans, baboons and mice are permissive. In immune animals, the immune response, mediated by a variety of antibody-dependent cell-mediated cytotoxic mechanisms, is directed against the migrating schistosomula and attrition occurs mainly in the skin and lungs. In attempting to unravel the response, it is highly desirable to have a laboratory model in which immunity in permissive and non-permissive hosts can be compared. Such a model appeared to be mice belonging to the 129 strain; in a proportion of animals, the life cycle progresses to the adult stage, whereas in the rest attrition occurs as a loss of worms from the portal system 3-4 weeks after infection^{4,5}. It now appears, however, that the outcome of the infection depends not on differences in immune responsiveness but on the architecture of the blood vessels associated with the lungs and liver¹⁻³.

It has been known for some time that the portal system of mice chronically infected with *S. mansoni* becomes 'leaky'⁶ and that this phenomenon can be investigated by injecting latex beads into a mesenteric vein. The technique has now been applied to uninfected 129 mice^{1,2}, and in a proportion of such animals the beads were not retained in the liver, as happens in most strains of mice, but became lodged in the lungs. Other studies confirmed the relocation of worms in the lungs of some

129 mice^{1,2,5}. It therefore seems that there is a way in which latex beads — and by implication worms — can migrate from the liver to the lungs. This problem was approached by making latex moulds of the vasculature of 129 mice^{2,3}. Experiments with the moulds showed that in some animals the vasculature was normal, but that in others there was a considerable truncation of the blood vessels of the liver and lungs which correlates with non-permissiveness³. The findings do not explain the migration of worms to the lungs. But an independent study, also using a latex mould², has shown that in permissive 129 mice there are connections between the hepatic portal system and the vena cava.

Three groups¹⁻³ have now demonstrated that the non-permissiveness of 129 mice is due to anatomical abnormalities and is not the result of an acquired immune response. But what actually happens? In normal mice the worms migrate to the liver and cannot get back to the lungs, whereas in some 129 mice they are able to do so, presumably through the connections demonstrated. Once in the lungs, there is a powerful antibody-dependent cell-mediated cytotoxic response, involving eosinophils, which kills the parasites and terminates the infection. The killing mechanism probably involves antibody

against schistosome glutathione-S-transferase, a mechanism that drew attention to the 129 mice in the first instance¹, although whether this is a cause or an effect of the parasite killing is not clear. Other possibilities such as the presence of Sendai virus¹, to which 129 mice are particularly sensitive, have now been ruled out¹.

The nature of the connections between the hepatic portal system and the vena cava have not been discussed, but the connections appear to correspond with the ductus venosus; in the embryo, the ductus venosus conveys blood from the umbilical cord directly to the common hepatic vein. Normally, this vessel disappears at birth but whether it persists in 129 mice and, if so, whether its persistence is correlated with the poor vasculature of the lungs and liver of these animals are questions yet to be answered. This series of observations, however, does serve as a warning not to postulate immunological mechanisms where simple anatomical or physiological explanations might suffice. □

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COMPUTING

In search of higher powers

Colin Upstill

THE solution of many important scientific problems requires computer power far beyond that offered by general-purpose machines. One way around this is to connect general-purpose processors together in a parallel computer; another is to build special-purpose hardware that achieves a particular computation as effectively as possible. Common examples of such hardware 'accelerators' include graphics co-processors, FFT (fast Fourier transform) engines, and, most recently, neural-network simulators. On page 33 of this issue, Sugimoto *et al.*¹ outline a hardware accelerator, called GRAPE (for gravity pipe), for force calculations for the gravitational many-body problem. Such calculations require enormous computer power: realistic simulations of the dynamics of astrophysical systems would take up to a teraflops year of calculation² (equivalent to a Vax 11/780 running for one million years).

Special-purpose hardware like that proposed by Sugimoto *et al.* can be used to

solve many other *N*-body problems, such as calculating Coulomb and van der Waals forces for molecular dynamics simulations. Many other types of scientific computation require vast computer power, for example, solving the equations of quantum chromodynamics, key to our fundamental understanding of the nature of matter. This sort of computation is only going to be feasible in the foreseeable future by using special-purpose hardware, parallel computing or both.

Advances in semiconductor technology have benefited both approaches. Sugimoto *et al.* propose a GRAPE chip using custom LSI (large-scale integration; tens of thousands of transistors per chip) clocked at 30 MHz. A multi-chip parallel processor could be realized fairly easily, because the many-body forces are simply additive. Today's ultimate hardware solution is custom VLSI (very-large-scale integration) clocked at many tens of MHz, which, with feature sizes down to 1 µm or less, can pack hundreds of thousands of

transistors on a single chip—enough for several GRAPE processors.

But semiconductor technology cannot be pushed that much further — certainly not by several orders of magnitude. Feature sizes are limited by quantum physics, and clock frequencies approaching 1 GHz correspond to cycle times of a few nanoseconds: electrical signals travel less than a metre in this time, posing serious design problems for computers of any physical size. The continual pursuit of ever greater computer performance will inevitably lead to new technologies being developed.

Recent advances in nonlinear optical materials and devices, largely driven by the rapid uptake of optics in telecommunications, have brought us to the threshold of the integration of optics with electronics in computing. One word sums up what optics means to computing — parallelism. Large numbers of light beams can cross the same region of space without interfering, and signals of different wavelengths can be sent simultaneously along the same path. Groups in Europe, Japan and the United States are developing algorithms, architectures and devices for both optoelectronic and all-optical computers. In the last few months, systems that can reasonably claim to be digital optical computers have been demonstrated independently at AT&T Bell Laboratories in the United States¹ and Heriot-Watt University, Edinburgh, in the UK². A colleague and I have recently designed an application-specific optoelectronic computer which would process images at the speed of one million Vaxes, using the fundamental operations of binary image algebra as its instruction set. Its feasibility has been verified by simulations on a 'conventional' electronic parallel computer, and such a computer can be developed to perform more general array processing³. The technology will exist to build this machine, and others like it, within the next three years: for example, it has been recognized for more than two decades that optics might provide the very large number of connections needed for neural networks, and a variety of proposals for optical neural computers are now being seriously pursued.

A problem with any hardware solution such as a GRAPE chip¹, is that it is extremely costly to debug: almost any error in design or implementation means re-engineering, in expensive contrast to editing an erroneous line of software. The technologies for programmable parallel computers are rapidly maturing, and can deliver gigaflop performance. Typical systems use reconfigurable arrays of off-the-shelf microprocessors, interconnected by switchable routing chips which are software-controlled. Such systems often have better cost/performance than either dedicated hardware or supercomputers,

Land yacht earns its wings



This winged "land yacht", designed by two Oregon inventors and the US National Aeronautics and Space Administration (NASA), finished second in the world last month at the World Cup Regatta international land sailing championship.

Inspired by the vertical-wing design of *Stars and Stripes*, the US catamaran that soundly defeated New Zealand in last year America's Cup race, the yacht uses a NASA-designed laminar-flow airfoil to reach speeds of nearly 90 m.p.h. in a 20 m.p.h. wind. Amateur inventors Philip and Art Rothrock collaborated on the project with engineer Harry Morgan of the Langley Research Station's Subsonic Aerodynamics Branch, incorporating three decades of NASA

research on low-drag aerodynamics.

With a laminar-flow airfoil, the boundary layer of moving air closest to the surface of the wing stays smooth and non-chaotic until it approaches the rear of the airfoil, much further back than in traditional airfoil designs. Because drag is proportional to the amount of wing surface over which airflow has become turbulent, laminar-flow wings typically demonstrate drag reductions of 30 per cent or more over traditional designs.

Because only the high end of the land yacht's performance falls within NASA's test data for low-speed, high-lift airfoils, much of the vehicle's final design has been worked out during trials on desert or dry-lake test courses.

G. Christopher Anderson

and enormously extend the realm of software solutions.

A promising new technology which blurs the distinction between hardware and software is configurable array logic, based on a model, such as a cellular automaton, rather than an instruction set. Implemented as an electronically reconfigurable array (a kind of generic integrated circuit, parts of which can be electrically modified after manufacture), 'programming' is a very low-level task, achieved by physically editing the content of control registers — not dissimilar to machine-code programming of microprocessors. The particular model implemented limits the uses of such a device, but, unlike conventional integrated circuits, debugging does not involve throwing them away and making new ones.

Special-purpose hardware like that proposed by Sugimoto *et al.* is neither inferior nor superior to either general-

purpose computers, or reconfigurable parallel computers or any other solution. Each approach trades ease of implementation and general applicability for cost/performance in different ways. The excitement of advances in both hardware and software solutions is that it is now becoming possible to tackle problems never previously attempted, not because we did not know how, but because they were not computationally feasible. □

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Variation among the masses

Gary J. Olsen

ANALYSIS of the composition of natural microbial populations is often restricted because the component organisms have to be grown in the laboratory. But by comparing molecules from the microbial environment, rather than the organisms themselves, this need for cultivation can be circumvented. In reports appearing on pages 60 and 63 of this issue, Giovannoni *et al.*¹ and Ward *et al.*² describe the characterization of the 16S ribosomal RNA (rRNA) sequences from naturally occurring biomasses using broadly applicable techniques. Although the groups examined entirely different environments, and used different procedures, the most important observations to emerge are strikingly similar. First, none of the organisms revealed by the molecular analyses are the same as any of those cultivated from the corresponding, or similar, environments, showing that the diversity in the natural populations is far greater than that demonstrated by cultivation-based methods. Second, the microbial populations seem to include distinct clusters of closely related species.

Distinctive characteristics

The identification of a microorganism requires the detection of its distinctive array of characteristics, a process that has traditionally required pure cultures. In some cases the detection of a particular molecule (for example an unusual lipid or chromophore) can be used as a marker for a specific group of organisms in a mixed population. But the availability of suitable markers is limited and they do not necessarily have the desired phylogenetic resolution.

In 1965 it was pointed out³ that the sequence of a nucleic acid or protein is much more evolutionarily informative than is knowledge about whether its activity is present or absent (usually inferred from the presence of a corresponding metabolic product). The identification of specific macromolecular sequences in natural biomass therefore provides a more precise and powerful characterization of a population than do inventories of lipids or chromophores. Sequences allow a quantitative definition of both the genetic diversity and the organismal relationship within a niche.

The use of ribosomal RNAs in identifying organisms and inferring their natural relationships, a field pioneered by Woese, has been especially valuable because these molecules are universally distributed, constant in function, slow to change in sequence and easily manipulated⁴. Ribosomal RNAs can also be used to characterize uncultivated organisms⁵.

Pace and colleagues recognized the potential of rRNA-based analysis of natural populations⁶. Early studies based on 5S rRNA demonstrated the principles, but were limited by the need to purify each molecular species and by the small size of 5S rRNA (about 120 nucleotides)⁵⁻⁷. Use of the more informative 16S rRNA (about 1,500 nucleotides) or 23S rRNA (about 2,900 nucleotides) came with the recognition that rRNA-specific primers could be used to sequence the larger molecules rapidly^{8,9}. A final obstacle, the searching of large libraries of cloned DNA from natural populations for rRNA genes, was overcome by applying rRNA-specific primers to the amplification of the desired sequences from bulk genomic DNA before cloning¹⁰.

Ward and colleagues² have now examined the 16S rRNAs from a well-studied photosynthetic microbial mat in a hot spring in Yellowstone National Park. Previously, the authors had sequenced the rRNAs from an assortment of bacteria cultured from the mat. In the new study, the 16S rRNA molecules from mat biomass were selectively copied (using a 16S rRNA-specific primer) into DNA, which was then inserted into a bacterial plasmid. Subsequent clonal purification of the plasmid replaced the traditional culture-based purification of the microorganisms. Eight distinct sequence types were represented in the 15 plasmid clones characterized. None of these rRNA sequences is the same as those from any of the 12 cultured prokaryotes believed to be associated with the mat. More significantly, in only one case does a sequence bear any close resemblance to a recognized bacterial 'phylum'. Unless the bias in the organisms represented by the cultures and the bias in the rRNA-based characterization are in opposite directions, it would seem that the species represented in culture are but a small fraction of the organisms in the mat.

Giovannoni and colleagues¹ looked at the 16S rRNA gene sequences of organisms from a sample of oligotrophic (low-nutrient) ocean water. The authors used the polymerase chain reaction to amplify the 16S rRNA gene sequences in their sample of bulk genomic DNA before cloning the DNA into a bacterial plasmid. The nine sequences obtained fall into two clusters, one related to cyanobacteria (oxygen-producing photosynthetic bacteria) and one to another main eubacterial group, the alpha purple bacteria⁴. Each cluster contains three distinct sequences. Although this work is not directly related to a culture-based study, D. Distel and J. Waterbury of the Woods Hole

Oceanographic Institute have determined 16S rRNA sequences of cyanobacteria isolated from similar environments (see ref. 1). The cultured cyanobacteria are not the same as any of those represented in the environmental samples, but two of them are clearly members of the same cluster (raising the number of distinct sequences in the group to five).

Natural diversity

The tentative conclusion that can be drawn from the two latest findings is that cultured organisms represent only a small proportion of the diversity in nature. Giovannoni *et al.* also emphasize the sequence diversity within their two clusters. Ward *et al.* uncovered seven disparate groups, only one of which has more than two sequences in it. This last group also shows sequence diversity (about three per cent sequence difference) comparable with that observed by Giovannoni *et al.* For perspective, this is comparable to the difference between the 16S rRNAs of *Escherichia coli* and *Salmonella typhimurium*.

These studies mark the advent of a new stage in microbial ecology. Neither group routinely determined complete 16S rRNA sequences, and so the precision of the inferred sequence relationships is not certain. Also, the sampling of population diversity is too small for confidence in the apparent trends; confirmation of the results might require the characterization of many more clones.

In the near future, selected microbial populations will no doubt be thoroughly investigated with molecular techniques. The resulting data will be essential for understanding such processes as genetic divergence, physiological divergence, migration and competition in the microbial world. In addition, the molecular data are directly applicable to the design of rRNA-based hybridization probes¹ and 'phylogenetic stains'¹¹, which can be used for more easily tracing the distributions of bacterial species or groups of species. □

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Comet orbits and chaos

Mark E. Bailey

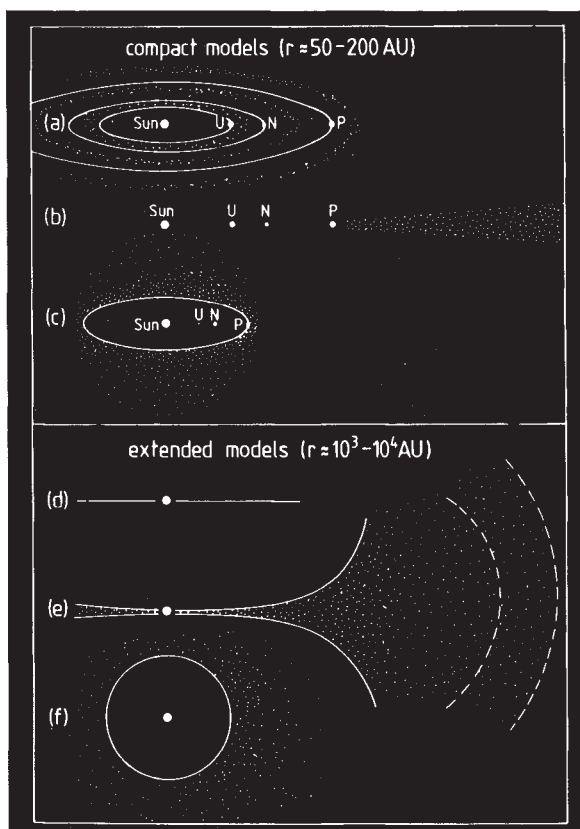
AT ONE time, the notion that comets are a by-product of planetary growth dominated ideas about their origin. According to the planetesimal hypothesis, for example, comet nuclei are presumed to have formed essentially through a two-step process: first, a rapid coagulation of ice-covered dust grains orbiting in the outer protoplanetary disk at heliocentric distances of the order of 10–30 astronomical units (AU); then, gravitational accumulation of these aggregates into larger bodies, 'planetesimals', and then into the growing planets themselves. The ice-covered dust must have been present in the protoplanetary disk, because otherwise it is difficult to understand the observed bulk composition of the outer planets. In recent years, however, a variety of alternative suggestions has been proposed, many tending to place comet formation further from the Sun — sometimes far from the presumed accretion zones of the main planets, thereby decoupling comet formation from that of planetary growth. Results described by Torbett and Smoluchowski on page 49 of this issue¹, implicating chaotic orbital dynamics in the problem, now give a further twist to the tale.

On the planetesimal theory, planetesimals (or comet nuclei, as they are presumed to be) aggregate through collisions into larger and larger bodies, eventually producing the observed set of outer planets. At the same time, those bodies that avoid collision with a growing planet suffer gradual changes in their orbital semi-major axes, some to larger and some to smaller values. Those scattered inwards, it is suggested, may contribute to the lunar 'late heavy bombardment'² (and hence to the volatile mass fraction of the inner planets, including the Earth³), whereas those sent outwards finally populate the region of the Sun's influence beyond Neptune and Pluto known as the Oort cloud or are ejected into interstellar space^{4,7}.

An attractive feature of this theory is that gravitational scattering of residual planetesimals leads to a more or less spherically symmetrical Oort cloud containing comets with orbits extending up to about 10^5 AU. Moreover, this 'observed' cloud⁸ is predicted to include a massive 'inner core', containing comets with semi-major axes ranging from around 10^3 AU to about 2×10^4 AU.

According to the theory, the comets

with semi-major axes near the bottom of this range should for the most part be moving on eccentric elliptical orbits inclined at a relatively small angle to the mean orbital plane of the planets. At larger semi-major axes (greater than $3-5 \times 10^3$ AU), external perturbations produce a more isotropic velocity distribution, eventually leading to an almost spherical inner core which merges imperceptibly into the observed outer Oort



Several models of the dense inner core of the Oort cloud (see text for explanation; taken from ref. 18).

cloud with semi-major axes greater than 2×10^4 AU (refs 7, 9).

Evidence for this outer cloud comes from the observed near-parabolic cometary flux. Indeed, Kazimirschack-Polonskaya and Everhart, among others^{10,11}, have often argued that the same near-parabolic flux (assumed to represent a steady state, but including comets with perihelia ranging up to about 30 AU) leads, through planetary perturbations, to the hundred or so observed short-period comets (periods less than 200 years). Most of them have relatively short periods ($P < 20$ years) and move chiefly in low-inclination orbits under the influence of Jupiter.

A number of authors¹²⁻¹⁶, however, have demonstrated two important difficulties with this explanation for short-

period comets: first, the capture probability from the observed near-parabolic flux is too small to explain the observed number; second, the predicted distribution of their orbital inclinations is too broad, unless cometary decay is significant. So it seems that many, if not all, short-period comets must come from another source, in particular (assuming a dense inner-core hypothesis) either from a spherically symmetrical inner core, or from a separate, perhaps highly flattened 'disk source'^{15,17}.

In fact, a variety of different, yet not necessarily mutually exclusive, pictures has emerged. Several proposed variations

on the dense inner core are shown in the figure, illustrating a convenient separation between so-called 'compact' and 'extended' inner-core models¹⁸. Whereas the compact models contain comets with semi-major axes typically in the range 50–200 AU (presumably formed on the outskirts of the protoplanetary disk, and therefore separate from the formation of planets), the extended ones contain comets with very much larger orbits. The latter are consistent with the predictions of the planetesimal hypothesis, but might also arise through formation of the Oort cloud *in situ*, possibly involving the accumulation of comets in a massive extension of the protoplanetary disk or during the original collapse of the protosolar nebula¹⁹.

Hitherto, although flattened, compact models of types (a) or (b) as shown in the figure have frequently been preferred as a source of short-period comets¹⁴, the precise means by which the original low-eccentricity, low-inclination orbits beyond Neptune are converted into suitable planet-crossing trajectories have remained uncertain. A resolution of this difficulty now seems possible from the discovery^{1,20}, that the orbits of comets in such a belt or disk are chaotic on time-scales as short as 10 million years.

Previously, it was suspected that comets formed well beyond the accretion zones of the main protoplanets at heliocentric distances greater than about 35 AU would move in regular, stable orbits for at least 4.5×10^9 years, the age of the Solar System. The new results now indicate the reverse: the range of 'chaotic' trajectories in the outer Solar System is surprisingly large; and provided the perihelia of such comets lie in the approximate range 30–45 AU, the accumulation of distant, weak perturbations by the four major planets can lead to orbits that eventually cross Neptune. Once this occurs, comets have a small, but significant probability of finally evolving into

observable short-period orbits under the control of Jupiter.

By finding a mechanism in which comets initially in orbits slightly beyond Neptune may evolve into observable short-period comets, the idea that a compact, flattened inner core may be the source of most observed short-period comets seems to be strengthened. On the one hand, the idea may be viewed as a mere extension of the planetesimal theory (albeit one in which most comets have originated on

the outskirts of the protoplanetary disk, and in which short-period and long-period comets now come from different parts of the Solar System, having experienced quite different histories). But, viewed from another perspective, there may now be a superfluity of proposed 'sources' to explain short-period comets. □

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CELL BIOLOGY

Motoring in the spindle

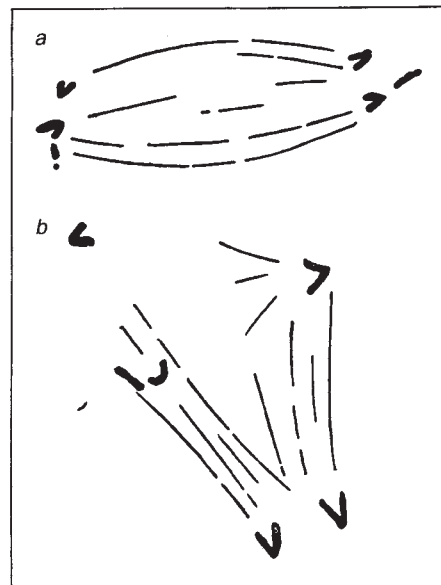
Ken Sawin and Tim Mitchison

It has long been thought that protein motors generating force on cytoplasmic microtubules have a part in spindle formation and chromosome movement during cell division. Until recently, such proteins were known to function only in rather specialized cell types — dynein in cilia and flagella, and kinesin in neurons. Microtubule-based motor proteins may have more diverse roles, but their involvement in mitosis has remained controversial. With the report by Endow *et al.* on page 81 of this issue¹, genetics may have come to the rescue, as has often been the case in complex systems.

Endow and colleagues report the nucleotide sequence of the *claret non-disjunctional* (*ca^{nd+}*) gene in *Drosophila melanogaster*. Oocytes of *Drosophila* females mutant at the *claret* locus have defective chromosome segregation during meiosis, resulting in offspring that are usually aneuploid and dead; sequence analysis of the *ca^{nd+}* gene now shows that its protein product is strikingly similar to the heavy chain of *Drosophila* kinesin, a well-characterized microtubule motor². This result is all the more exciting in light of two reports in *Cell* describing the identification of kinesin-like genes with mitotic defects in other organisms, the *KAR3* gene in the yeast *Saccharomyces cerevisiae*³ and the *bimC* gene in *Aspergillus nidulans*⁴. The sequence similarity among these four genes shows that kinesin is the prototype of a family of microtubule motor proteins, and the phenotypes of the three mutants indicate that at least some members of the family are important in mitosis.

The kinesin heavy chain, sufficient for generating microtubule motility *in vitro*, has an amino-terminal domain that binds microtubules and ATP and generates force by ATP hydrolysis. This motor domain is linked through a parallel α -helical coiled-coil stalk to a carboxy-terminal globular region thought to interact with motile elements such as vesicles or organelles. The *ca^{nd+}*, *KAR3* and *bimC* gene products contain regions highly similar to the kinesin motor domain, but outside this domain all four proteins are different. As well as indicating important functional regions within the motor domain, the sequence similarities in the kinesin family have already been used to identify new members by way of the polymerase chain reaction. This route identified a kinesin-like gene from *Drosophila* that turned out to be the same as the *ca^{nd+}* gene⁵. This molecular approach will no doubt be helpful for identifying kinesin-related genes in other eukaryotes, especially those refractory to classical genetic analysis. The odds are stacked in favour of such proteins being microtubule motors and, like kinesin, plus-end-directed (see below), although this must be tested directly *in vitro*. What may be different among these proteins are the substrates to which they bind — which determine biological function — and perhaps the rates at which the motors move along microtubules.

What roles might we expect microtubule motors to have in the mitotic spindle? The function of a given motor is dependent on the direction of its inter-



Histological preparations of *Drosophila simulans* oocyte meiotic spindles. *a*, Control oocyte, late anaphase of meiosis I; *b*, *claret* mutant at the same stage. The abnormalities which have been described include multipolar (as shown), unipolar and multiple spindles. From Wald (ref. 9).

action with microtubules, which are inherently polar structures. In the spindle, microtubule 'minus' ends are at spindle poles and 'plus' ends are attached to chromosomes at the kinetochore. Moving chromosomes towards the spindle pole in anaphase therefore requires a minus-end-directed motor, unlikely to be the *ca^{nd+}* gene product or any member of the kinesin family (readers are advised to watch this space). As Endow *et al.* point out, the movement of chromosomes away from poles as they form the metaphase plate may also require a motor protein bound to the chromosome at the kinetochore. Indeed, a plus-end-directed microtubule motor has been found in the kinetochores of isolated chromosomes using functional assays⁶.

But possible functions for plus-end-directed motors in mitosis are not limited to those bound to chromosomes. In *Aspergillus bimC* mutants, spindle pole bodies fail to separate after reproducing and mutant cells fail to form bipolar spindles. Yeast mutants lacking the *KAR3* gene product are primarily defective in the microtubule-based nuclear fusion that follows conjugative mating, but also seem to be retarded (but not arrested) in mitosis with a short spindle. Both of these mitotic defects can be explained by microtubules of opposite polarity failing to slide apart. Spindle pole separation driven by the sliding apart of half spindles is required both in spindle formation and in anaphase B spindle elongation. These movements are thought to be driven by a plus-end-directed motor or motors⁷.

What is the function of the *ca^{nd+}* gene product? As it is probably a plus-end-

directed motor, it might be part of the kinetochore, responsible for chromosome alignment on the metaphase plate, or part of the spindle, responsible for microtubule sliding during spindle morphogenesis or anaphase B spindle elongation. Because ca^{nd+} messenger RNA is found mainly in oocytes⁸, the protein would have one of these roles in the female meiotic spindle. Chromosome loss and non-disjunction would be expected from mutants in either process. Whether the ca^{nd+} gene product is a kinetochore protein or a general spindle protein will probably be decided by its cytological location once antibodies are available.

In the meantime it is interesting to speculate about the function of the ca^{nd+} gene product on the basis of its mutant phenotype and sequence. Based on *KAR3* and *bimC* experiments, a role in spindle morphogenesis and expansion is the most likely. Cytological observations of mutant meiotic spindles reveal global defects in microtubule organization rather than malpositioning of individual chromosomes (see figure), supporting the notion that ca^{nd+} encodes a microtubule sliding protein and not a kinetochore protein. Endow *et al.*, however, favour the idea that ca^{nd+} encodes a chromosomal, probably a kinetochore protein, for the following reason. Surviving offspring of *claret* mutants are often genetic mosaics, indicating that chromosome non-disjunction or loss can occur in early mitotic divisions as well as in meiosis.

Surprisingly, mitotic chromosome loss is specific for maternal chromosomes. The lack of ca^{nd+} mRNA in testes suggests that its role in meiosis must be filled by a different protein in males, but why should maternal and paternal chromosomes be differentially affected once they are in the same cytoplasm? The authors propose that the *claret* phenotype is transmitted with maternal chromosomes, even when the ca^{nd} gene product is absent (that is, in the null mutant ca^{nd}). This might mean that the ca^{nd+} gene product binds to the maternal chromosomes and segregates to their progeny after replication, while a different gene product performs the same role for paternal chromosomes. This argument seems to be based on genetic data, independent of the nature of the ca^{nd+} gene product. With the finding that the ca^{nd+} gene product is a microtubule motor, Endow *et al.* say that its most likely chromosomal location is the kinetochore. The idea that kinetochore proteins segregate to daughter chromosomes without exchanging with a cytoplasmic pool is intriguing. In general, there is still much to be learned about the fate of tightly bound chromosomal proteins during DNA replication.

Does the maternal specificity of the mitotic effect of *claret* mutations rule out a non-chromosomal location for the protein

encoded by ca^{nd+} ? We think not. Over 50 years ago Wald⁹ proposed that the mosaic phenotype results from loss of chromosomes during meiosis, followed by resorption of these chromosomes into some of the daughter nuclei. Perhaps more likely is a model in which the meiotic spindle organizational defect is inherited by the maternal portion of the first mitotic spindle. The first mitotic division is unusual in *Drosophila*, in that the maternal and paternal chromosomes seem to organize early spindle structures independently and do not actually intermingle until mitosis is complete. So some structural component of the maternal meiotic spindle could be important for organizing the maternal half of the first mitotic spindle. It follows that the maternal half of the mitotic spindle could inherit an aberrant structure from a mutant meiotic

COSMOLOGY

A case of extended inflation

David Lindley

COSMOLOGICAL inflation is a beautiful picture mounted in an ugly frame. At the heart of all the many and varied versions of inflation is an appealingly simple mechanism that, in principle, explains why the Universe, which began in a Big Bang of microscopic dimensions (about 10^{-4} m), has managed to grow into the huge and relatively uniform (as indicated by the smoothness of the microwave background) volume we now inhabit. But if the fundamental idea is appealing, the theories that have been invented to make it work as desired are definitely not. The subterfuges resorted to by particle physicists and cosmologists in their attempts to construct 'successful' inflationary models are often so complex and arbitrary that one has a nostalgia for the old days, when the present appearance and properties of the Universe were summarily ascribed to initial conditions of mysterious provenance. But on page 47 of this issue, Paul Steinhardt¹ describes a new version of inflation which, if one is willing to accept a time-varying 'constant' of gravity, dispenses with many other complications and may thus restore some of the lost simplicity of inflationary cosmology.

The central idea behind all versions of inflation remains the simple one first proposed by Guth². The popular notion of symmetry breaking in particle physics, by which first the strong interaction and then the weak and electromagnetic forces are split off from a single interaction as the characteristic energy of particles (in this case, the temperature of the cooling Universe) decreases, relies on something called the Higgs mechanism. In simple terms, imagine a ball rolling about on an

spindle, producing maternal-specific mitotic defects. One candidate for such an aberrant heritable structure is the centrosome, or microtubule organizing centre, which is possibly defective in *claret* mutant oocytes¹⁰. □

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uneven surface: it will find the lowest point, which becomes the vacuum state of the theory, and the small oscillations of the ball about that stable state (their energy, amplitude and period, for example) define the lowest-order excited states, which represent various physical particles.

In the Higgs mechanism, the shape of the uneven surface is made to be a function of the energy of the system, so that, as the Universe cools, some previously stable valleys, where the vacuum state could reside, become unstable, and new ones arise. When the ball rolls from one position to another, wholesale changes in the phenomenology of the vacuum state occur. The nature of the small oscillations changes, meaning that one set of particle masses and properties is replaced by another. And interactions mediated by these particles suddenly look different, which is how the Higgs mechanism makes different forces from a single theory.

When the vacuum state changes, its absolute height above some zero of energy changes too. Guth's observation was that in a cosmological setting this height translates into a non-zero vacuum energy density, which can alternatively be thought of as a positive cosmological constant. If this constant energy density ever comes to dominate other contributions to the density of the Universe (from the kinetic energy of particles and radiation), the Universe will expand exponentially with time. This has a number of benefits: a tiny volume of the pre-inflation Universe, which could reasonably be expected to be fairly uniform, is blown up into something of macroscopic size, explaining why the visible Universe today looks roughly the

same everywhere; in addition, an exponentially expanding Universe tends asymptotically towards a 'flat' cosmology, whose density approaches the critical density separating universes that expand forever from those that recollapse, whereas a conventional universe filled with radiation and particles diverges away from flatness. Inflation, therefore, was able to explain in principle how the Universe could have lived so long, yet still be near the critical density and be reasonably uniform.

The difficulty is that the Universe, after going through the exponential inflationary phase, must return to something that looks like a conventional universe of matter and radiation. In Guth's original proposal — old inflation — two Higgs vacuum states were separated by a finite energy barrier, and inflation occurred because the Universe got stuck in the higher of the two states. Guth then assumed that quantum tunnelling through the barrier to the low (zero energy density vacuum) state would bring the Universe back to normality. This certainly can happen, but what it creates is an expanding bubble of 'true' vacuum separated from a sea of 'false' vacuum by a material wall (as in a soap bubble) in which energy and particles reside. Old inflation failed because, in the return from the inflationary to the conventional phase, the appearance of these bubbles created a wildly inhomogeneous universe and erased all the advantages of inflation.

Then came 'new inflation'^{3,4} in which the barrier between false and true vacua was made to be tiny, and the vacuum state rolled unimpeded from false to true vacuum during the period of exponential expansion. But it could roll neither too fast, or inflation would be curtailed before it could do its job, nor too slowly, or the failings of old inflation would reappear. Moreover, as the vacuum state rolled across an almost flat potential energy surface, small place-to-place variations in its position tended to grow, which led to cosmological density variations: these were desirable, in that they might lead to galaxy formation, but their scale and magnitude were sensitively dependent on the rate of rolling.

New inflation worked, but only if the form of the potential energy surface was just so: a small industry briefly flourished as people tried to construct suitable potentials from supersymmetric or technicolour or Kaluza-Klein theories. The besetting problem was that the construction of suitable potentials required so much fine-tuning and adjustment of parameters that it was hard to believe that nature would be so kind; the whole point of inflation was to avoid fine-tuning cosmological initial conditions, but new inflation put the fine-tuning in the particle physics instead.

What Steinhardt suggests is almost a return to old inflation. In his model of 'extended inflation', the barrier between true and false vacuum is finite again, and quantum tunnelling is what ends inflation. But the new ingredient is a variation of the strength of gravity, coupled somehow to the cosmological expansion. This adds a new term to the conventional Friedman equation relating expansion rate to energy density, and in effect stretches out inflation from an exponential expansion into a mere power law. The extended period of power-law expansion accomplishes the same goals as exponential inflation, although it takes longer. But at this slower pace, bubbles of true vacuum, when they emerge, also expand more slowly, and are able to mix and homogenize the material content of the Universe quickly enough to establish a 'normal' Universe in time for nucleosynthesis, for example, to take place.

Steinhardt's proposal may have another bonus. Old and new inflation both predict that the density of the Universe today can differ from the critical value by only an exponentially small amount. Astronomers, unfortunately, persist in believing that their observations indicate a cosmological density of probably about half the critical value. Some enthusiasts for inflation insist that the cosmological observations are wrong, or at least incomplete; others resort to more fine-tuning to coax a subcritical density from an inflationary theory. But extended inflation offers another way out. Again by virtue of its power law rather than exponential expansion, critical density is approached more slowly than in old or new inflation, which means that a small, but not ridiculously small, range of initial conditions can lead to a Universe with density something less than critical.

The bugbears of modern cosmology are fine-tuning and tooth fairies — Michael Turner's name for theoretical ideas which turn up in the nick of time to save a cosmological model from demise. Steinhardt's extended inflation throws out a lot of fine-tuning, but brings in one new tooth fairy in the guise of a variation of gravity. But the gravity variation does not, apparently, have to follow any particular functional form (so we are not dealing with a finely-tuned tooth fairy), and gravity variation is a tooth fairy of some maturity, having first been seen by Eddington and Dirac. According to these semi-quantitative criteria, therefore, extended inflation looks more credible than most of its rivals. □

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Imperial raiment

MODERN thin-film technology is dominated by vapour-deposition methods. But Daedalus has other ideas. He recalls the old trick of dissolving two chemical precursors of nylon in two immiscible solvents. When the solvents are mixed, they react at their interface to form a nylon film, which can be pulled continuously out of the mixture. DREADCO's chemists are using solutions so dilute, and pulling them from the mixture so fast, that the resulting polymer film is exceedingly thin — much less than a wavelength of light.

The most obvious commercial product is a multi-film laminate. By passing the initial film through a sequence of further dual solvent baths, each adding a layer of a different polymer, a strong and useful multilayered film can be built up. Like float glass, the film retains the optical perfection of the liquid surfaces that formed it. With proper choice of the thickness and refractive index of each layer, an interference filter results: strong, flexible, cheap, as large as you like, and with any desired optical properties.

Already DREADCO's tailors are forming this optical fabric into various 'Emperor's New Clothes'. Some, designed for minimum optical reflection, are practically invisible, but reflect and retain the body's infrared for warmth and comfort. Others combine mirror-like reflectivity in the visible region with total infrared transparency, for maximum heat rejection and cooling in tropical climates. Yet others show the wonderful richness and depth of pure colour that only a multilayer filter can give, or the iridescent rainbow patterns shown by thin soap films.

More appealing still, if the solutions are contacted not as layers but in emulsion form, and the solvent inside the resulting coated droplets is allowed to diffuse out and evaporate, the remaining microballoons form a decorative optical dust with the elusive interference structure of the opal.

With sufficiently dilute solutions, the final film approaches a monolayer, only one molecule thick. Like ultrafine polymer monofilament, it is remarkably strong; yet it is totally invisible. Bulk invisible film is very hard to handle, so the DREADCO workers are concentrating on the emulsion-polymerization form of the process. A powder of hollow monolayer microballoons can be handled and poured like a sort of invisible heavy gas. As the ultimate expanded polymer, it is a wonderful thermal insulator, and the only one transparent enough to be placed between the panes of double-glazed windows. Daedalus hopes to carbonize it into the long-sought spherical hollow graphite macromolecules, of which only the smallest possible example, the C₆₀ prototype, has so far been persuasively demonstrated.

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Beta radiation from snow

SIR—We have detected radioactive snow in a 6-metre-deep snowpit at a site 38 kilometres northeast of the South Pole. We would like to report here our ideas about the cause and significance of this beta-radiation.

We have examined the depth profile of samples collected at 5-cm intervals from the pit, and we find an annual signal in the beta-radioactivity profile during 1955 to 1974, presumably resulting from the intrusion of stratospheric air into the troposphere each summer. But we found an unexpected peak in beta-radioactivity in samples at 10–20-cm depth (see figure).

Examination of the profiles of Na^+ , Cl^- , Cl^-/Na^+ and of H_2O_2 indicate that the depth interval 10–20-cm represents snow deposited in the summer of 1987–88. Gamma-spectrometric analysis shows that ^{137}Cs , at levels of 0.23 ± 0.20 and $1.25 \pm 0.11 \text{ pCi kg}^{-1}$ (in the 10–15 and 15–20 cm samples, respectively), is responsible for the elevated beta-radioactivity.

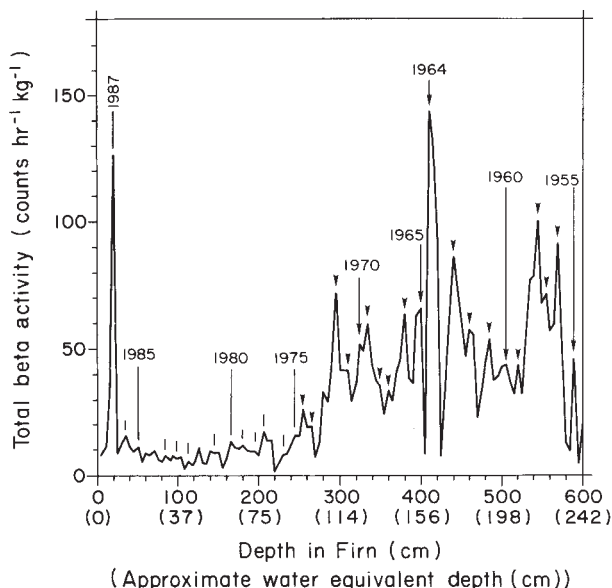
The source of the ^{137}Cs is problematic. The Chernobyl reactor accident in 1986 could be one explanation; deposition of debris 20 months after the accident is in good agreement with the lag in bomb fallout from Northern Hemisphere atmospheric tests. But there is no ^{134}Cs , the characteristic fallout isotope from Chernobyl, in the labelled layer. The relatively small samples from the top of the snowpit make detection and quantification of radioactivity at these levels difficult, so an explanation involving Chernobyl cannot be excluded.

If Chernobyl debris was deposited at the South Pole, it must have been transported in the stratosphere. Although most of the radioactive material from Chernobyl remained in the troposphere, some of the debris did penetrate the tropopause. If there was Chernobyl debris in the stratosphere, why was no fallout reported in the Southern Hemisphere?

The apparent restriction of recent radioactive fallout in Antarctica (and perhaps the entire Southern Hemisphere) to the region near South Pole suggests that the polar vortex may have focused stratospheric fallout onto the central Antarctic plateau. There is pronounced

subsidence of stratospheric air within the vortex of the Antarctic ozone hole, although it is not clear that this air descends to the surface.

The concentration of the cosmogenic radionuclide ^7Be in Antarctic aerosol has been shown to be a tracer of stratospheric air reaching the surface. Although the



Depth profile of total beta-radioactivity in the snowpit, sampled in December 1988. The unexpected peak in beta-radioactivity at 10–20-cm depth results from the presence of ^{137}Cs .

relationship between aerosol and snow concentrations of ^7Be have not been firmly established, a limited data set of ^7Be concentrations in Antarctic snow indicates much greater stratospheric influence in the centre of the Antarctic polar vortex.

The presence of Chernobyl debris in the snow near the South Pole would have important implications about atmospheric transport to the Antarctic Plateau, and could provide a valuable new reference horizon for researchers working in this region. But our results, from a single snowpit, are far from conclusive. The preservation of Chernobyl debris on the Greenland ice sheet is very uneven, and the ^{137}Cs snow near the South Pole is probably at least as patchily distributed, suggesting that very careful sampling of a number of pits is needed to quantify the amount of ^{137}Cs that has recently been delivered to this remote area.

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Magnetic isotope effect

SIR—The magnetic isotope effect — the dependence of the rate of chemical interactions between radicals on the spins and magnetic moments of their nuclei — is a remarkable phenomenon¹. It results in the selection and redistribution of magnetic and non-magnetic isotopes among the different products of radical reactions. The effect is known for ^{13}C (refs 2,3), ^{17}O (ref. 4) and ^{29}Si (ref. 5). Most recently⁵, it has been claimed for ^{235}U . We have now measured the effect for ^{33}S .

The photolysis of phenyl-acyl-phenyl-sulphone ($\text{PhCOCH}_2\text{SO}_2\text{Ph}$, or **I**) in SDS micelles is accompanied by the separation of sulphur isotopes: the starting ketone **I** is enriched in the heavy isotopes ^{33}S (2.4%) and ^{34}S (0.6%) with respect to the light non-magnetic isotope ^{32}S and about 80% conversion of **I**. The enrichment of non-magnetic ^{34}S nuclei is probably due to the classical mass-dependent isotope effect, however, the measured enrichment of the magnetic isotope ^{33}S is four times that of ^{34}S . ^{33}S enrichment would be expected to be less than that of ^{34}S if the mass-dependent effect were the sole cause.

We suggest that this discrepancy is explained by the magnetic properties of ^{33}S , whose nuclei have spin 3/2 and a magnetic moment of 0.643. The immediate product of photolysis of **I** will be the radical pair PhCOCH_2 and SO_2Ph in the triplet electronspin state. Triplet-singlet intersystem crossing governed by electron-nuclear hyperfine interaction will induce recombination of the ketone **I**, but the hyperfine interaction of the magnetic ^{33}S nuclei will accelerate triplet-singlet conversion of radical pairs in which it is contained, increasing its abundance in the regenerated ketone. On the other hand, triplet-singlet conversion of radical pairs containing the non-magnetic sulphur nuclei ^{34}S and ^{32}S will be less rapid, with the result that radical pairs containing these nuclei will more often be transformed into products other than **I**.

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Autoimmune response in AIDS

SIR—The viral protein Nef has an important role in the often long latency period of infection by the human immunodeficiency virus HIV-1. Antibodies raised against Nef have been detected in otherwise seronegative individuals¹ and T-cell mediated responses to Nef have been reported². Even during the latency period, when only

complete β -chain sequences contained in the SWISS-PROT database and in 14 of the 16 complete HIV-1 Nef sequences contained in the human retrovirus and AIDS database (November 1989). The exceptions are the Z321 Nef, where there is only one mismatch, and the Mal Nef, where there is an apparent deletion at

confirmed, suggest early therapeutic intervention aimed at neutralizing the adverse effects of Nef during the latent phase of the disease.

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Drought resistance in rice

SIR—Drought is a major problem for much of the world's rice crop. Of the 146 million hectares of land on which rice is grown, only one half is irrigated. In the rest, which accounts for one-quarter of rice-grain production, yields are often depressed by drought^{1,2}. Moreover, in many irrigated rice soils, hard pans block root growth and so impede water extraction³.

The impact of drought on crop production can be reduced by developing varieties better adapted to water deficits⁴. Despite this, drought resistance in rice has historically had a low priority in breeding programmes². As in many other crops⁵, two causes of this are lack of basic understanding of the mechanisms of drought injury and resistance, and want of ready means to connect physiological and biochemical knowledge with genetics and breeding. Participants at a recent workshop⁶ considered how plant biotechnology could help to relieve these constraints. The following conclusions were reached.

(1) Especially for tropical rices, there is an urgent need to understand the plant better, in relation to drought at various life-cycle stages, and to physiological, biochemical and molecular levels of organization.

(2) The genetic material and the research expertise available in the international rice research network in Asia are great resources. Building bridges between this network and plant scientists in other countries could help to speed the development of new tools for drought resistance breeding, and to advance basic knowledge of water stress responses in rice.

(3) New breeding tools would be provided primarily by incorporating selected physiological traits into the restriction-fragment length polymorphisms program being developed by the Rockefeller Foundation's International Program on Rice Biotechnology⁷. An example would be the genes governing deep rooting/

a

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Nef-Bru 33 ASRDLEKKGATSSNTA ATNACAWT EAQEEVEVGFETVQVPLRMTYKAADVLSHFLKEKGGLEG
DQ- $\beta$  137 RTEALNHNHLLICSVDFYPSCHKVRWRNDQET TGVVSTPLIRNGDNTFQILVML EMTFQPGDVYT
DR- $\beta$  105 KTQPLQHNLVYVSFCFYPSCHKVRWRNDQET TGVVSTPLIRNGDNTFQILVML EMTFQPGDVYT
DP- $\beta$  133 KKGFLQHNLVYVSFCFYPSCHKVRWRNDQET TGVVSTPLIRNGDNTFQILVML EMTFQPGDVYT
      * * * * *

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b

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L..H..[ILV]..{13,15}W[FL]..{2,4}QE[DE]..{2,5}G.....[ILV]...[MW]T[YF].....[ILV]..{6,7}G
[ENQ][HK]

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a, Amino-acid sequences of an HIV-1 Nef protein and HLA class II antigen DQ, DR and DP β -chains are displayed showing the region of similarity. Asterisks, positions containing common or conservative amino acids in all HLA class II β -chains and HIV-1 Nef sequences (except Mal). The # sign indicates an additional set conserved in all but a single unusual HLA class II DO β -chain. Boxes indicate the postulated β strands modelled on the α 3 domain of the HLA class I protein. b, Common pattern to HLA class II β -chain and Nef HIV-1 pattern expressed as a regular expression. Dot, any amino acid; capital letters in square brackets, conserved alternative amino acids; curly brackets, range of a variable spacer or gap. (Further details available on request from T.F.S.)

a small proportion of peripheral blood lymphocytes (1 in 10^4 – 10^5) appear to express viral structural messenger RNA, a progressive impairment of the immune defences is observed^{3,4}. This last observation is compatible with an autoimmune response as part of the progression of disease. We have found a common structural pattern in the sequences of Nef and the β -chain of the HLA class II histocompatibility antigens which could induce such an autoimmune response.

The HIV-1 Nef proteins share a region of 60–70 amino acids which is highly conserved. A search of the SWISS-PROT protein sequence database (release 12, EMBL, 1989) reveals that this region is similar to the immunoglobulin-like second extracellular domain of the β -chain of the HLA class II histocompatibility antigens (see figure). Within this region, a common diagnostic pattern can be identified such that no other known proteins in this region are compatible with the postulated HLA class β -chain structure modelled on the known α 3 domain of the HLA class I protein.

We identified this common pattern using an iterative multialignment pattern induction algorithm⁵, and find it is statistically equivalent to sharing more than 10 identical amino acids. The sequence contains some of the amino acids thought to be essential for the HLA class II β -chain fold and most of the common amino acids are distributed proximally on the surface of the HLA class II β -chain/ α 3 domain model structure. (We chose the placement of gaps in the pattern to be compatible with the modelled structure.) The pattern appears in all HLA class II

position 61–63, removing an otherwise conserved tryptophan. The pattern is not found in HIV-2 or simian immunodeficiency virus sequences equivalent to Nef.

The existence of this common structural pattern and the early generation of antibodies against Nef suggests a mechanism for an autoimmune response against the HLA class II β -chain induced by HIV-1 Nef through 'molecular mimicry'. This possibility is further supported by reports^{6,7} of antibodies against self-HLA class II molecules and lymphocytes in individuals infected with HIV-1. It is relevant that animals injected with antibodies directed against their constitutive class II histocompatibility molecules develop a severe immunodeficiency comparable to that present in the HIV-infected human individuals.

Finally, the absence of the HLA/Nef common pattern in HIV-2 Nef is consistent with the greatly reduced pathogenicity observed for this subtype. An immune response against Nef during the latent phase of the infection, resulting in an autoimmune response against HLA class II molecules, could lead to the progressive deterioration of the immune system. This impairment appears to allow eventual active replication, leading to the late stages of AIDS. These hypotheses, if

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water extraction and penetration of compacted soil layers⁸. Once identified and mapped, these genes (which are extremely difficult to score in the field) could be readily tracked in a breeding programme via the associated molecular markers.

(4) Advances in basic knowledge of stress responses could come first from using the RFLP map in an analytical way to dissect major components of drought resistance, and second by creating novel variation by genetic transformation. An example of the former would be the identification of independent sets of genes controlling the same complex trait, such as genes regulating embryo abortion or pollen infertility⁹, both of which cause drought stress-induced sterility. More knowledge is needed to design and produce agronomically useful genotypes by transformation techniques, but some valuable experimental approaches, such as transfer of genes for betaine synthesis¹⁰ into rice can be imagined.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □

Gaian test

SIR—Lovelock (*Nature* **344**, 100; 1990) is unhappy at the lack of acceptance of his Gaian hypothesis but adds nothing that would help to answer any of the legitimate questions which arise. Instead, he ignores most of the wisdom of the past 300 years while shooting himself in the foot.

His first error is his table of 'successful predictions'. Long ago, David Hume pointed out that no number of positive instances 'prove' anything; the homely instance is that of a child dropping a soft toy from its pram a hundred times; this provides no guidance whatsoever for the experience of heaving a rubber ball overboard.

Popper expresses the idea explicitly. The essential property of a scientific theory is not verifiability, but falsifiability. The problem of Gaia is that it is a 'theory of everything'. As such, it is capable only of self-fulfilling prophecies. But Lovelock provides us in his article with a strong testable assertion. "Among the insights that come from a Gaian approach are that planetary life can never be sparse. A planet with a sparse life can never be self-regulating."

But the geological record indicates a number of catastrophes, each involving the disappearance of fossilizable species to at least 90 per cent. Clearly 10 per cent survival is not 'sparse'. Should a geological horizon be discovered tomorrow involving 99 per cent extinction, then 1 per cent is not sparse.

The assertion, which looked like a good test, merely demonstrates non-falsifiability, and hypotheses which are as resistant as this to contradiction are not scientific or philosophic, they are religious. No wonder Lovelock can boast that he is "moved by the interest taken by theologians and philosophers", while deploring the "rubbish" which the theory has accreted.

Lovelock's second error is to try to extract value and purpose from the theory, that is to derive a value from a fact (even if we agreed about the facts), a valuation from a description, or an 'ought' from an 'is'. As Hume put it, "In every system of morality, which I have hitherto met with I have always remarked, that the author proceeds for some time in the ordinary way of reasoning, and establishes the being of a God, or makes observation concerning human affairs; when of a sudden I am surprised to find that instead of the usual copulations of propositions is and is not, I meet with no proposition which is not connected with ought and ought not . . .".

Lovelock insists that Gaia (his replacement of one God by another would have made Hume smile), provides guidance for "understanding the consequences of pollution and environmental disturbance".

This idea is again testable. From the evidence or facts of the geological record we can see that repeated extinctions over the last 500 million years have eliminated 90 per cent of species without disruption to long term gaian self-regulation.

We conclude that we may proceed likewise, and proceed with extinctions, exploitations and pollution up to the 90 per cent level, without either physical or moral danger, and since we are not to be treated as special, we may be included in the 90 per cent. This is the logical conclusion. Is it acceptable to Lovelock or his theological, philosophical or green adherents? I think not.

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Ischaemic injury mediator

SIR—Feigl has suggested¹ that nitric oxide ($\cdot\text{NO}$), derived from endothelium, may be an important detoxifying mechanism for superoxide (O_2^-). Although O_2^- and $\cdot\text{NO}$ are known² to form rapidly the peroxynitrite anion, ONOO^- , our results suggest that ONOO^- is toxic and that preventing its formation may explain the therapeutic efficacy of circulating superoxide dismutase in many diverse insults³.

Peroxyntitrite has a pK_a of 6.8 at 37 °C and when protonated decomposes to generate highly reactive agents: apparently hydroxyl radical ($\text{HO}\cdot$) and nitrogen dioxide (NO_2). Moreover, ONOO^- has a half-life of under 1 second at pH 7.4, allowing it to diffuse to critical cellular targets before decomposing. We have shown that classical $\cdot\text{OH}$ scavengers competitively inhibit oxidation initiated by ONOO^- decomposition, while the iron chelator desferrioxamine is a potent scavenger because of its direct reaction with ONOO^- , independently of its ability to bind iron.

Macrophages and neutrophils produce both O_2^- and $\cdot\text{NO}$ when activated^{4,5}, suggesting that ONOO^- may be an additional cytotoxic agent formed by these cell types. In endothelium and brain, Ca^{2+} is the intracellular messenger initiating the NADPH-dependent oxidation of arginine to produce $\cdot\text{NO}$ (ref. 5). Pathophysiological processes such as ischaemia, excessive activation of the *N*-methyl-D-aspartic acid receptor in brain, and sepsis allow the influx of Ca^{2+} into cells, which may stimulate the simultaneous production of $\cdot\text{NO}$ and O_2^- . Injurious amounts of ONOO^- could result, because every ten-fold increase in the concentrations of $\cdot\text{NO}$ and O_2^- will increase ONOO^- formation 100-fold.

Such a mechanism may be important for reperfusion injury to ischaemic tissue, as ischaemic endothelium will accumu-

late Ca^{2+} and the substrates (arginine and NADPH) needed for NO synthesis but will produce $\cdot\text{NO}$ or O_2^- only when oxygen is supplied by reperfusion. Intravenously administered superoxide dismutase may reduce ischaemic injury by scavenging O_2^- before it reacts with $\cdot\text{NO}$ to form ONOO^- .

This hypothesis is consistent with current data showing the reduction of ischaemic injury by superoxide dismutase, desferrioxamine and $\cdot\text{OH}$ scavengers, but it also predicts that increased amounts of nitrate, nitrite and nitrosamines should be detectable in ischaemic tissue and its venous drainage.

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To flow or not to flow?

SIR—Bretscher, in a recent Scientific Correspondence¹, has questioned some of the conclusions drawn in our recent articles^{2,3} and renewed his advocacy of his lipid-flow hypothesis. We had shown that in a stationary reference frame, cell surface proteins labelled with small gold beads could be in either of two states: randomly diffusing or systematically transporting rearwards toward the cell centre. Because there was no detectable systematic drift of the diffusing proteins, we concluded that lipid flow does not drive the motion either of the diffusing or of the transported particles.

One question of Bretscher's arose from a misunderstanding concerning the velocity of rearwards movement of cell surface components relative to forward motion of the cell. He suggested that the apparently randomly diffusing particles were in fact moving backwards on the cell surface because the cells were moving forward at a comparable rate. In our study, however, forward motion of the cells was much slower (20–100-fold) than the rearwards transport of surface components. Hence, the absence of rearward drift of surface particles observed in the laboratory reference frame also holds for reference frames fixed to the cells. In a later study of rapidly moving cells⁴, we have also observed that the diffusing particles do not display systematic transport relative to the cell even after the cell has migrated several cell lengths.

The second question Bretscher raises was whether our particles were really on

the surface of the cell. The strongest evidence that they were is that 0.5-micrometre beads, which can clearly be shown to be on the upper surface of the lamellipodium by varying the focal plane of the microscope, also do not move toward the rear when they are diffusing. There are additional arguments that the viscosity of cytoplasm in the lamellipodium would not permit such rapid diffusion and we have documented similar diffusive behaviour of gold particles which were subsequently released from the membrane into the medium. Further, direct measurement of lipid labels in a photobleaching experiment has detected no rearward movement of bulk lipid even though the cells were moving forward at 40 micrometres per minute⁵.

As in the case of the fabled unicorn, there may be somewhere where lipid flow can be found, but we have not seen evidence for it in motile cells.

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Myelination in severed nerves

SIR—There are other explanations for the interesting observation by Voyvodic¹ of increased myelination in the sympathetic nerve innervating the submandibular gland in rats, than those implied by his title "Target size regulates calibre and myelination of sympathetic axons". He says that "relative target size was increased by cutting one branch of the sympathetic nerve innervating the gland at birth", but no details are given as to which branch was cut, or how.

In 1900 Langley² made the surprising discovery that, after excision of the right superior cervical ganglion in the cat, the submandibular sympathetic nerve trunks on the submandibular artery did not disappear, as expected, and that one year later "there were more medullated fibres, and apparently fewer non-medullated, in the nerve strands on the artery on the right side than in those on the left — the intact side". He found no evidence for any functional reinnervation, and said that he would leave the cause of the regeneration for further investigation.

Some years ago, I followed up this unusual observation. I found that after superior cervical ganglionectomy in cats,

although the non-myelinated axons had degenerated in the sympathetic trunks on the submandibular artery by day 12, some myelinated axons always persisted³. Subsequently, it was found⁴ that some myelinated axons were present at all stages after ganglionectomy and that their numbers increased with time up to one year. Despite the drastic procedure of sectioning the post-ganglionic sympathetic trunks, together with ligature and transection of the associated external carotid artery — just proximal to the submandibular artery — some myelinated axons in the trunks on the submandibular artery always survived and in time they increased in numbers.

The source of the myelinated axons in the submandibular trunks that withstand such nearby axotomies remains a mystery. Sometimes, small axons with newly forming myelin were seen after a few weeks close to myelinated axons of more normal size, so they may have arisen from sprouting in the vicinity, alternatively *de novo* myelination may have been occurring.

Histochemical assessments of cholinesterase activity in the sympathetic nerve trunk on the submandibular artery, after post-ganglionic axotomy⁵, are of interest. Normally, these sympathetic trunks show very weak acetylcholinesterase staining and somewhat stronger non-specific cholinesterase activity⁶; this is the converse of the reaction in post-ganglionic parasympathetic nerves, which show very strong acetylcholinesterase activity. After superior cervical ganglionectomy, there is an early loss of all cholinesterase staining in the submandibular sympathetic trunks, followed by a gradual increase, especially of acetylcholinesterase activity. In other words, the submandibular sympathetic trunks come histochemically to resemble parasympathetic nerves. Since these changes were initially more evident closer to the gland, it was suggested (ref. 5) that the unmyelinated axons in the trunks had arisen from collateral sprouting of cholinergic post-ganglionic parasympathetic nerves within the gland, which had then migrated in a retrograde manner down the sympathetic trunks.

Much work remains to be done to ascertain the true cause of any increase in the number of myelinated nerves in the sympathetic trunks to the submandibular glands after denervation experiments.

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Ever-decreasing circles

Chris Brand

The Descent of Mind: The Nature and Purpose of Intelligence. By Peter Evans and Geoff Deehan. Grafton Books: 1990. Pp. 206. £14.95.

THIS popularly written book draws its curiously appropriate title from an idea which is a favourite of the authors, that human intelligence is somehow especially 'social' in its nature and evolutionary origins. Yet in fact the book's coverage ranges fairly widely across familiar modern concerns about intelligence. Does intelligence have an identifiable basis in the functioning of the nervous system? Can it be improved? Is it already rising across generations? And will *Computer sapiens* ever join the club? (The nature-nurture question is not covered here: this controversy is said on page 39 to have "disappeared" following what the authors take to be the discrediting of Sir Cyril Burt.)

Apart from the copious use of blank pages, the book's most novel feature is the study technique that is employed. In universities in the United Kingdom, nobody can telephone colleagues, especially international colleagues, for a chat — certainly not before lunchtime, nor pay them for their advice, let alone visit them to check out their laboratories, unless already well-embarked on a corporate research-organizing raid on taxpayers'

pockets. By contrast, the British Broadcasting Corporation, the organization for which these authors work, cuts through all this red tape and eschews the universities' diversion of funds into teaching, learning and assessment centres. Thus is science-at-the-frontiers-of-knowledge delivered to BBC audiences and, here, to readers. So the authors present the very latest sayings of a few dozen modern researchers of intelligence and IQ and, at more notable length, the views of Howard Gardner, Nicholas Humphrey, Marvin Minsky and Robert Sternberg.

It would be unfair to say that discipline is entirely lacking from the exercise. As BBC professionals, the authors have been well-drilled in the need to maintain a semblance of balance. So, with the help of well-chosen quotations from competing experts, they never stay on any one tack for long. Any pro-IQ sentence is swiftly followed by an anti-IQ paragraph; computers are not allowed to think or fall in love for more than a page; and even the authors' general optimism about "headstart" programmes is qualified by a one-sentence admission that some of the leading proponents of the "Miracle

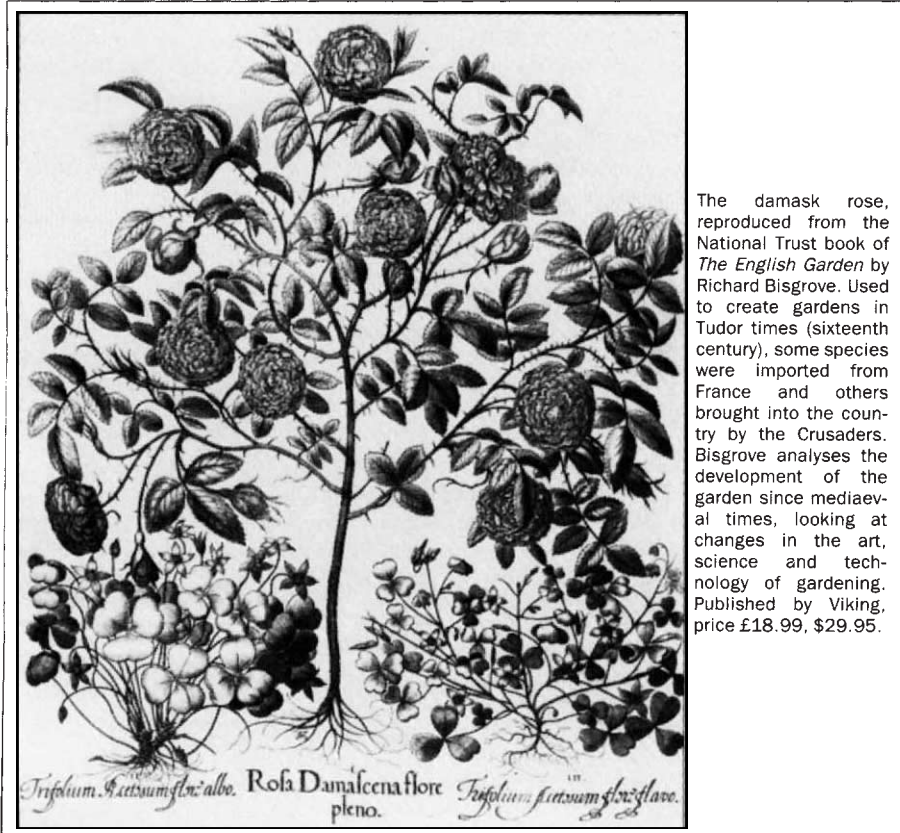
in Milwaukee" finally ended in jail.

Nevertheless, there is a discipline problem. Despite their reflexive balancing acts, the authors repeatedly reveal their own preference for a multidimensional view of intelligence: they clearly like Gardner's idea that there are at least seven quite independent types of mental ability. It is perfectly proper for the authors to entertain such a thought. So did the great US psychometrician, L. L. Thurstone, in the 1930s. Yet Thurstone and his intellectual successor, the mighty J. P. Guilford, who managed to believe in 150 distinct abilities, felt obliged to try to provide empirical evidence for the view. It is in the clash of these titans with other scientists, like A. R. Jensen and H. J. Eysenck, who note that human abilities (when reliably measured) correlate positively in population samples, that we find the chief substance of our century's enduring argument about intelligence.

Thus one can only greet with amazement the absence from *The Descent of Mind* of any reference to these four major players of the great IQ game. It may seem pedantic to expect the appearance of the names themselves; or to expect even half-serious discussion of what the authors call "the abstruse statistical technique of 'factor analysis'". Yet the absence even of correlation coefficients from the book must give pause. Had the authors asked Ian Deary, when they interviewed him, what was the correlation between IQ and inspection time (a tachistoscopic measure of perceptual intake speed), he would probably have said it was "around -0.50", for that is indeed the well-attested (if not undisputed) estimate that emerges from about forty studies to date. By contrast, had the authors asked Gardner for his empirically achieved correlations between his theoretically independent abilities, it is doubtful whether Gardner would have been able to give the required reply of "around zero" any more than he does to his own publishers.

Sadly, the discipline of asking scientists for their correlations is spurned by the BBC. So Evans and Deehan settle for Gardner's claim that his seven 'independent' abilities are "firmly rooted in the mechanics of the brain" — without offering a shred of evidence for this claim either. Such will be the disappointments for scientific readers of this no-expense-spared survey of all the latest about intelligence.

Do the authors resolve any better their own favourite problem of whether intelligence is somehow communal in its 'descent'? Well, no. They are impressed by Humphrey's idea that we have all been evolutionarily selected to read and sympathize with each other's minds. This is, apparently, so that we can "attract mates and produce offspring". For better or worse, however, fertility differentials



The damask rose, reproduced from the National Trust book of *The English Garden* by Richard Bisgrove. Used to create gardens in Tudor times (sixteenth century), some species were imported from France and others brought into the country by the Crusaders. Bisgrove analyses the development of the garden since mediaeval times, looking at changes in the art, science and technology of gardening. Published by Viking, price £18.99, \$29.95.

across studiable history lend no compelling support to this theory. Intelligence confers no clear breeding advantage upon its possessors: many of us manage our biological business quite adequately without enjoying a high IQ. As to the envisaged romantic and/or marital meeting of minds, one recalls the rueful observation of the Canadian prime minister, Lester Pearson: "Behind every great man there stands an astonished woman." Rather more seriously, the authors do not reflect that mind-reading capacities would be of no evolutionary advantage unless and until there were some passably interesting minds to read: the logical fallacy of attributing our intellectual descent to 'mind-reading' appears to be that of *petitio principii*.

Most strangely, despite their preference for a 'social' view of intelligence, Evans

and Deehan offer no evolutionary hypothesis as to why people differ so much in intelligence. Could it be that intelligence differences are maintained in human communities because they enable social stratification, hierarchy, leadership and division of labour? Such a hypothesis may not seem so friendly, but it is just as 'social' in its way, and singularly congruent with our distinctive human organization into tribes, nations and firms that require and provide for people of very different levels of mental ability. Here, at least, in whether human patterns of intelligence are crucial to *Homo hierarchicus*, is a question to which Evans and Deehan could usefully devote their next survey of off-the-cuff, expert opinion. □

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One of the crowd

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Biochemistry By C. K. Mathews and K. E. van Holde. Benjamin Cummings: 1990. Pp. 1096. £24.95.

TO REVIEW a textbook of general biochemistry (which in this case comprises 1,129 pages) is never an easy task. In the short time allowed for perusal only a superficial judgement can be reached concerning the quality of the product and, inevitably, certain sections are subjected to a more detailed scrutiny than others. Here, an immediate and sincere response is one of unqualified admiration for the two authors who have somehow found the time, dedication and patience even to contemplate the idea of writing this book.

What were the driving forces behind their decision? There is no doubt that Mathews and van Holde can reap rich financial rewards if their text becomes recommended in even a few of the many scientific departments throughout the world. But the competition is fierce. Already, excellent textbooks of biochemistry abound, for example, *Biochemistry* by Voet and Voet published this year by Wiley. Furthermore, the 3rd edition of *Biochemistry* by Stryer (Freeman) — a consistently first-class text — appeared as recently as 1988. Mathews and van Holde claim that a motivation to write in their case arose from a desire to produce an ideal, contemporary biochemical text for teachers. Ultimately, of course, the success or otherwise of their effort will be judged by the response of the student population itself.

The text is very readable throughout and the facts are, in the main, presented

with commendable clarity. The style is fresh and enthusiasm for the subject abounds. Purists will undoubtedly cringe at phrases such as "freeways on the metabolic road map", "on-ramps from the highways and byways of stage 1 and stage 2 metabolism" and "The mitochondrion — scene of the action". Nevertheless, the authors make the points in question with refreshing clarity, and students seem unlikely to protest.

The diagrams are generally very good and there is a pleasing combination of computer graphics with more traditional illustrations. Indeed, some of the figures are outstanding. I particularly liked the use of 'swinging arms' to clarify the functioning of the pyruvate dehydrogenase complex — always a difficult mechanism for undergraduate students to digest. The organization of the text shows much thought and there is successful inclusion of the essential concepts of physical biochemistry and a generally well-balanced integrated coverage of biosynthetic and degradative reactions.

Without doubt, one of the real successes is the inclusion of a series of excellent sections on "tools of biochemistry". Far too many science undergraduates (and graduates for that matter) fail to appreciate or understand the experimental basis underlying the development of sophisticated biochemical concepts. It is far too easy to take facts for granted. The methodology sections are real plus points and help to generate much more interest from the main text. They will aid students in their overall comprehension of biochemical facts, both simple and complex, and they will reinforce too the often-neglected point that progress in science increasingly depends upon the development of new techniques, many of which display exquisite applications of simplicity and logic.

Despite my overall enthusiasm for this textbook, I do have some criticisms. In-

evitably, some sections are stronger than others and certain chapters fail to excite despite their soundness. The index entry for pyruvate carboxylase directs the reader to one page only, where activation of the enzyme by acetyl-CoA is dealt with. One has to look up "anaplerotic sequences" in the index to locate the (different) page that discusses this important enzyme in more detail. Commendably, the authors discuss ribozymes and correctly consider that RNA molecules might, and indeed do, have catalytic functions. This is a really exciting research field and I would have liked Mathews and van Holde to have developed it in relation to ribosomal RNA, which might well turn out to be the main determinant of ribosomal functions.

Reflecting my own research interests, I was disappointed to find only a superficial coverage of antibiotics, although a short section entitled "the uses of biochemistry" stresses therein the exciting prospect of creating designer drugs. Penicillin flickers briefly in the text but I could find no reference to penicillin-binding proteins. It is a pity too, in my opinion, that Mathews and van Holde did not end each of their chapters with a summary section. From my own experience of teaching, I find that the average biochemistry student likes to be told in as concise a way as possible what are considered to be the main points in a given subject area.

All in all, however, *Biochemistry* is an excellent effort and I have enjoyed reading my copy. I wish the authors every success and I certainly expect to see a copy of the text on our library shelf. It will be interesting to see if our biochemistry students adopt this book for their main source of reference. □

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New Journals

This year *Nature's* annual new journals review supplement will appear in the issue of 11 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

■ Journals which first appeared after June 1988, and which issued at least four separate numbers by the end of April 1990, will be considered for review. The deadline for submission is the end of June.

■ Journals covering any aspect of science are eligible, though those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.

■ Frequency of publication must be at least three times a year.

■ The main language used must be English. Translation journals in English are eligible.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title as soon as possible and before the end of July to: Book Review Editor, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK.

Out of the blue

Scott Tremaine

Catastrophes and Evolution: Astro-nomical Foundations. Edited by S. V. M. Clube. Cambridge University Press: 1989. Pp.239. £25, \$44.50.

GEOLOGISTS and astronomers attempt to deduce the origin and evolution of the Earth and the Universe from their present states. Difficulties arise because astronomical and geological timescales are so long that observations provide only a single snapshot of complex time-dependent processes. To help interpret this snapshot, both disciplines rely on the paradigm of uniformitarianism, which asserts that natural laws are constant in space and time, and that past evolution should be explained through presently observable processes.

A weakness in this paradigm is that some important processes could occur rarely or last for brief periods, so that evolution proceeds mostly through a series of rare 'catastrophes' rather than at a steady rate. Two examples of proposed geological catastrophes, of very different degrees of plausibility, are the biblical deluge and the impact of a large extra-terrestrial body at the Cretaceous/Tertiary boundary. The theme of this book, a collection of lectures from a meeting of the British Association for the Advancement of Science held in 1988, is the conflict between uniformitarianism and catastrophism in geology, and the astronomical evidence that catastrophic events such as impacts by comets and meteors have been significant in geological and biological evolution.

A desirable goal for a book of this kind would be to provide an introduction for geologists to the relevant astronomical issues and vice versa. In this respect, there are several excellent articles here. Hallam provides a good historical survey of catastrophism in geology; Grieve's article is a short but authoritative review of terrestrial impact cratering; and Alvarez *et al.* give a brief, clear and provocative account of the current status of the hypothesis that the Cretaceous/Tertiary extinction resulted from a large impact. But the coverage of issues is uneven and somewhat parochial: one example is that there are no articles that make the case against the impact hypothesis for the Cretaceous/Tertiary extinction; another is that the discussion of astronomical issues strongly reflects the views of the editor and his collaborators. The general reader would not know that Clube argues for is a radically unconventional world-view, in which comets are captured from star-forming regions, giant comets with more than 100,000 times the mass of observed

comets populate the Solar System with dust and debris, redshifts may be gravitational rather than cosmological, and even general relativity and Friedmann models of the Universe are suspect.

To some extent, uneven coverage is inevitable in such a short book: the text

million years by the environmental consequences of a large impact, then each of us has roughly a one-in-a-million chance of dying from an unpredictable and unpreventable catastrophe of this kind. My local power company informs me that this is the same as the chance that I will be killed by



Halley's comet in 1065, 'a portent pregnant . . . with dangers and disasters soon to come.'

covers only 240 pages, the page size is small and the typeface is large. Given this limitation, *Catastrophes and Evolution* provides a useful and readable but in-complete introduction to the role of astronomical catastrophes in geological and biological evolution.

None of the authors addresses one interesting aspect of catastrophism. If most organisms are killed once every 100

cancer caused by a major accident if I live within 8 kilometres of a nuclear reactor, or that I will be killed on my next airplane flight. In a certain sense, astronomical catastrophes provide a natural and immutable lower bound to the riskiness of human life. □

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Sound reading

David Young

Arthropod Bioacoustics: Neurobiology and Behaviour. By Arthur W. Ewing. Edinburgh University Press: 1989. Pp.260. £27.50.

COMMUNICATION by sound in insects is now a thriving field of study, thanks mainly to the advent of convenient equipment for recording and analysing sound. Moreover, this is a form of behaviour that can be studied fruitfully at several different levels, from single nerve cells to selfish genes. I recently visited a laboratory where graduate students in neighbouring rooms were studying the neurobiology, ethology and physics of sound communication in a single group of insects.

Such combined studies have yielded some impressive results over the past couple of decades. In field and bush crickets (Orthoptera), for example, it has

been possible to show which song features are important in natural behaviour and to find some of the neurons responsible for processing these features in the peripheral and central nervous systems. The neural and mechanical devices by which the specific song features are generated in the first place have also been elucidated. Being able to study acoustic behaviour at these different levels not only adds depth to the research but also makes it eminently suitable for inclusion in undergraduate courses on animal behaviour.

It is therefore good to see relevant aspects of the subject brought together in this book by Arthur Ewing, who is an experienced researcher on insect song. The word arthropod is used in the book's title, but in the nature of things the book is almost entirely about insects, except for the occasional interjection from shrimp or scorpion. Bioacoustics is interpreted broadly, as hinted at in the subtitle, with the range of topics covered moving from the physics of sound to the genetics and evolution of insect song.

Anyone who undertakes to survey a field like this faces something of a dilemma. The traditional method, which is adopted by Ewing, arranges the subject by research technique: biophysics, sensory physiology, motor physiology, ethology and evolution. On the one hand, this makes it easy to compare species and to draw out general principles within each subject area. On the other, it separates things that clearly belong together, namely the behaviour and physiology of a group of insects seen in the context of their natural environment. Thus, anyone seeking a rounded picture of acoustic communication in field crickets would need to quarry in seven different chapters.

Essentially, what is offered is a comprehensive survey of recent research, presented in a way that should make it accessible to both graduate students and undergraduates. The chapters that top and tail the book should be particularly useful to the beginner. Some aspects of the physics of sound that are relevant to insects are outlined in the first chapter, where the main terms are defined. The last chapter describes methods for recording and analysing sound. The illustrations showing the same sound displayed as an oscillogram, a sonagram and a power spectrum are very helpful for the uninitiated, and might have been better at the start than at the end.

In between, there is a catalogue of research results: the physical mechanisms of sound production and reception, the

neural mechanisms of hearing and singing, then acoustic behaviour and the functions of calling, courtship and aggressive songs. Finally, genetics and evolution get the nod. Each group of insects receives its fair share of attention, depending on the amount of research that has been done. This means that the Orthoptera are the dominant group, but others such as cicadas and the author's own favourite, *Drosophila*, are not neglected.

The coverage of each subject is clear, accurate and thoughtful. It is not complete, however, and those interested in particular areas will need to search other recent references to get the full picture. This problem is most evident in the behavioural chapters on the calling and courtship songs of insects, particularly of crickets and cicadas. But the reference list provides an up-to-date guide to the literature, and even expert readers are likely to find some entries new to them.

Altogether, then, Ewing's book is a welcome contribution, the more so as it is the first of its kind since Haskell's *Insect Sounds* a generation ago. A comparison of the two books shows how much progress has been made. The breadth of coverage that Ewing has been able to maintain is particularly welcome. Even allowing for the present rate of progress, *Arthropod Bioacoustics* should be a valuable source-book for several years to come. □

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and ill-defined sketches of the outcome of palaeopedological research in this area.

One palaeosol type for which there is an abundance of information is the peaty soil (called entisol and histosol in the US system). But Retallack again fails to exploit this resource and his attempt to define the timescales of peat accumulation on the basis of peat thickness is positively misleading. To suggest that a peat depth of under 40 centimetres indicates an age of less than 800 years is entirely unacceptable. If Retallack had spent a little time in explaining the process of peat formation, including litter formation, detritivore and microbial activity, hydrological interactions and compaction effects, then it would soon become apparent that there can be no simple relationship between depth and age. There is evidence of a degree of oversimplification both here and elsewhere in his treatment of the interpretation of fossil soils.

The final section of the book, which is potentially the most interesting, is the story of the Earth's history as seen through the pores of the soil. In fact, the story begins beyond the Earth, on the Moon, Venus and Mars, with an account of totally abiotic soils. We are then led through the development of early terrestrial soils and the impact upon these of the rising oxygen levels in the primitive atmosphere. Immediately we hit upon the problem of a total lack of modern analogues: Hartley was right, the past is indeed a foreign country. It is difficult, therefore, on the basis of soil evidence, to determine the oxygen component of the Precambrian atmosphere.

The development of land vegetation provided a new soil-forming factor, and some palaeosols, such as coals, are an important source of information on plant palaeoecology. But again Retallack fails to deal adequately with the reconstruction of conditions in the coal-forming swamps (or bogs?) of the Carboniferous. We are left with no clear idea of the ecological, climatic or hydrological conditions in those palaeohabitats where coal ultimately accumulated.

The greatest strength of this book is in the clarity of its diagrams and Retallack's graphic explanation of soil-forming processes and soil classification systems. Its weakness lies in the author's failure to relate specific details of soil study to the broad process of environmental history. The complexity of soil terminology often seems to contribute to this lack of clarity, statements becoming buried and obscured by jargon. Whether or not things were done differently there, visiting the soils of the past certainly demands a foreign language. □

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Foreign visit

Peter D. Moore

Soils of the Past: An Introduction to Palaeopedology. By G. J. Retallack. *Unwin Hyman*: 1990. Pp. 520. Hbk £60; pbk £24.95.

THE past, according to novelist L. P. Hartley, is a foreign country — they do things differently there. But it is the ardent hope of palaeoecologists (a group including the palaeopedologists) that they did things much the same, so that we can confidently use the present as a key to understanding the past. For this reason, any book on palaeosols needs to begin with a section on modern soils and soil processes before it can adequately discuss the soils of the past. Here, Retallack provides a graphic description of three classification systems of modern soils — the traditional Australian system, the US system (with its hierarchical structure and distinctive language), and the rather hybrid FAO system. Retallack compares these in diagrammatic form and, together with discussions of soil structure and soil-forming processes, provides a secure basis

for the analysis of the yet more complex problems of describing and interpreting fossil soils.

Time is involved at two levels in the study of palaeosols: in the initial development and maturation of the soil, and in an extended period during which the soil lies in a 'fossil' state. Interpretation of a palaeosol entails the separation of these two processes. Post-burial diagenesis often involves compaction and, at high temperature, may even involve metamorphosis of the soil. Cementation and mineral replacement can take a soil far from its original, pre-burial state. Confusion of the two timescales is possible, as the author shows by examples from the Carboniferous and the Permian.

Yet some clues to a soil's original condition remain intact, such as the nature of the organisms, or their traces, left within the palaeosol. From fungi to termites, and from nematodes to mammals, a vast range of biological information lies within the fossil soil and provides some secure evidence of past climates and conditions. On the basis of such information it should be possible to reconstruct whole ecosystems of the past from a fossil soil, but here Retallack is disappointingly brief and superficial, supplying only vague

A special-purpose computer for gravitational many-body problems

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A processor has been constructed using a 'pipeline' architecture to simulate many-body systems with long-range forces. It has a speed equivalent to 120 megaflops, and the architecture can be readily parallelized to make teraflop machines a feasible possibility. The machine can be adapted to study molecular dynamics, plasma dynamics and astrophysical hydrodynamics with only minor modifications.

THE gravitational many-body (or N -body) problem consists of N point-mass particles moving under mutual gravitation according to Newton's equations of motion. It is relevant to several astrophysical problems. For example, if the particle represents a star (or a galaxy), the N -body system corresponds to a star cluster or an elliptical galaxy (or a cluster of galaxies or a large-scale structural feature of the Universe).

Many simulations of this sort have been performed, but they are highly computer intensive because of the need to calculate the gravitational interaction between $N(N-1)/2$ pairs of particles. Consider the case of the dynamical evolution of a globular cluster. To simulate its core collapse, the subsequent formation of binary stars in the collapsed core and the ensuing expansion of the core, teraflops (10^{12} floating-point operations per second) years of computing power are required if N is to have realistic values ($\sim 10^5$)¹. So far such simulations have been performed only with at most $N \approx 1,000$ – $3,000$.

Nevertheless, such self-gravitating many-body systems are of great interest not only because they are found commonly in astrophysical problems but also at a fundamental level: such a system has no state of final thermodynamic equilibrium. As the system evolves, structures appear spontaneously in the density distribution. To describe such structures realistically we need greater numbers of particles and thus more computing power.

To reduce the amount of computation, other methods for calculating the gravitational field have been considered². One example is the fast Fourier transform (FFT) for a spatially defined mesh; another is the grouping of particles that are distant from the one under consideration by use of tree algorithms. Both of these methods require of the order of $N \ln N$ operations to calculate gravitational interactions, rather than N^2 for the direct summation of gravitational forces over all pairs of particles. The FFT is very effective when (and only when) the necessary spatial mesh is uniform. Calculation of three-dimensional structures with very high density contrast does not meet this requirement. The tree algorithms are too complicated to extract the peak performance of supercomputers or of fine-grained parallel machines such as the Connection Machine³. Direct pairwise summation of forces, on the other hand, although placing excessive computational demands, can be adapted to any physical structure.

Recently the direct-summation approach has become more feasible because of the availability of highly integrated VLSI

(very-large-scale integration) chips. Our special-purpose computer is constructed relatively easily using such chips. The problem is to develop an architecture that allows both ease of construction and sufficient flexibility to simulate a wide variety of physical systems. The N -body problem is in fact well suited to these apparently conflicting demands. The simplicity is reflected in the computer's cost effectiveness; our prototype machine GRAPE-1 achieved a performance comparable to the CRAY-XMP/1 at a cost 10,000 times lower.

The pipeline architecture

The N -body problem is described by the equation

$$\frac{d^2 \mathbf{x}_i}{dt^2} = \mathbf{f}_i = \sum_j \frac{m_j (\mathbf{x}_j - \mathbf{x}_i)}{(r_{ij}^2 + \epsilon^2)^{3/2}} \quad (1)$$

where $\mathbf{x}_i$ and m_i are the position and the mass of the i th particle, $\mathbf{f}_i$ is the force exerted on it, r_{ij} is the distance between the i th and the j th particles, and ϵ is a softening parameter that avoids divergence of the force at $r_{ij} = 0$.

The computation is divided into two parts, one performed on a host computer (a workstation, for example) and the other on a special-purpose back-end processor. We name the latter GRAPE (for gravity pipe); it calculates the force $\mathbf{f}_i$ according to equation (1), through a 'pipeline' (Fig. 1). Other calculations such as the counting of i and advancement of the timestep are performed on the host computer. During one time step, the host

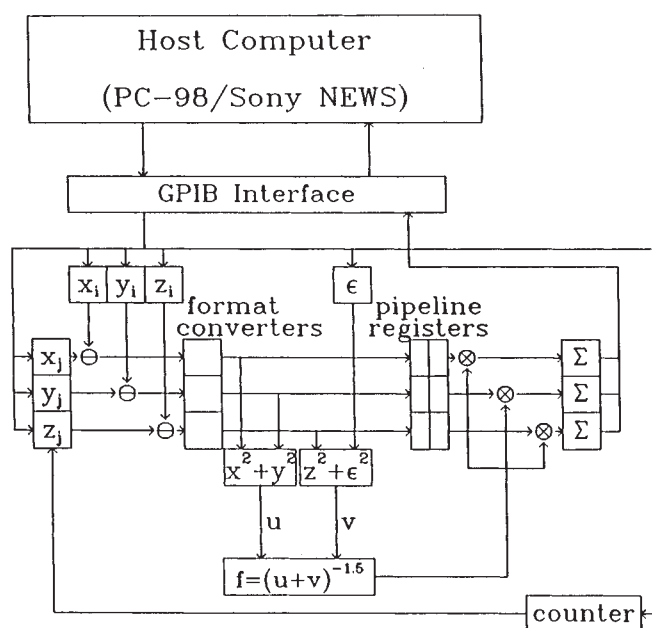


FIG. 1 Block diagram of GRAPE. Data flow along the pipeline successively for calculation of the force $\mathbf{f}_i$.

computer performs of the order of N ($O(N)$) operations whereas GRAPE performs $O(N^2)$. Communication between the two units requires only $O(N)$ operations, thus avoiding a communication bottleneck.

As seen in Fig. 1, the coordinates at time t —that is, $\mathbf{x}_j(t)$ (and $\mathbf{m}_j$) for all particles ($j = 1, \dots, N$)—are stored in GRAPE. The host specifies the particle i for which the force is to be calculated, and sends GRAPE its coordinates $\mathbf{x}_i(t)$. The x , y and z components of $\mathbf{x}_j(t)$ flow through the corresponding routes of the pipeline and an operation is performed on each of them during one internal clock cycle. The coordinates $\mathbf{x}_j(t)$ enter into the pipeline successively from $j = 1$ to N , j advancing by one in each cycle. Thus, the force $\mathbf{f}_i$ is calculated in $N + D$ cycles, where D (~ 10) is the latency of the pipeline. The resultant $\mathbf{f}_i$ is sent back to the host, which then advances the value of i to the next particle. When the forces have been calculated for all i , the host advances the time to $t + \Delta t$ and calculates $\mathbf{x}_i(t + \Delta t)$. The values of $\mathbf{x}_j$ are updated in the host and sent to GRAPE.

Applegate *et al.*⁴ have built a special-purpose computer, the Digital Orrery, for a small- N system such as the Solar System. The Digital Orrery has a parallel architecture in which small computers are interconnected in a ring. Each processor handles one particle. They did not adopt the pipeline architecture because it is inefficient for small N as a result of the latency. The pipeline is more efficient than the parallel architecture of the Orrery, however, when N is sufficiently large.

The two regimes of the N -body problem

There are two characteristic timescales in N -body systems. One is the crossing time t_{cr} , the time taken for an average particle to cross the characteristic size of the system. The other is the two-body relaxation time t_{rel} , the time taken for an average particle to change its energy through successive distant encounters (multiple scattering) and close encounters between particles. The ratio of these timescales is⁵

$$\frac{t_{\text{rel}}}{t_{\text{cr}}} \approx \frac{N}{11 \ln(0.4N)} \quad (2)$$

In most systems, $t_{\text{cr}} < t_{\text{rel}}$.

N -body problems are divided into two regimes according to the time span, t_s , over which simulations are made. The system is denoted collision-free or collisional according to whether $t_s \approx t_{\text{cr}}$ or $t_s \approx t_{\text{rel}}$, respectively.

For large systems, such as a galaxy or a supercluster of galaxies, the value of N is very large and t_{cr} is very long. Therefore, t_{rel} can be much longer than the age of the Universe, so that the systems are always collision-free.

For smaller systems, such as globular clusters, this is not always the case. For example, a binary globular cluster as observed in Magellanic clouds merges into a single globular cluster in 10^7 yr, and this process can be simulated as the evolution of a collision-free system⁶. But if longer timescales are considered, two-body relaxation becomes important. The core of a single globular cluster contracts gravothermally, and binary star systems are formed in the resultant dense core. This takes place over a timescale of the order of 10^9 yr and must be simulated as a collisional system^{1,7}. Thus a binary globular cluster has phases of both collision-free and collisional evolution.

The relation between the collision-free and collisional evolution is stated, in general, as follows. If the initial state of the system is far from virial equilibrium, the gravitational field changes markedly over a timescale t_{cr} . The distribution function undergoes strong mixing in phase space, and the global structure of the system is mixed in configuration space, both within the same timescale. This process is called violent relaxation and represents collision-free evolution. When violent relaxation has ceased, the evolution of the system becomes much slower and, if N is not too large, can be regarded as collisional.

Collision-free systems

Consider the simulation of a system with N_0 constituents which is represented by an N -particle numerical system. Thus one particle represents N_0/N constituents. This overestimates the effects of two-body relaxation relative to violent relaxation. Makino *et al.*⁹ have compared these errors with those due to the finite number of significant bits in GRAPE. They find that only about 4 or 5 significant bits are required for studying collision-free systems. Because the necessary number of effective bits is independent of N , a GRAPE device may indeed be devised with such a small number of bits. We must analyse carefully, however, the way in which the designated accuracy can be attained, because higher accuracies are required in some parts of the pipeline than in others.

These considerations led us to construct the machine GRAPE-1 (sw), details of which will be published elsewhere¹⁰. (Here sw signifies that the machine uses short words.) Because of the small number of bits handled in this machine, some functions of the type $w = f(u, v)$ can be incorporated as tables written on a single or several 512-kbit EPROM chips. It has a computing speed equivalent to 120 Mflops, 'equivalent' in the sense that the words are shorter in some parts of the machine than in supercomputers; calculation of the force between one pair of particles is estimated to correspond to 30 floating-point operations.

Because of the short effective digits, this machine can be used only for collision-free systems having neither a high-density core nor knots. As an example, we used GRAPE-1 to simulate the merging of a binary globular cluster with $N = 4,096$. We compared the results with those we obtained previously for the same physical system⁶ using an ETA-10 supercomputer. The global characteristics of the results, but not the positions and velocities of each individual particle, are practically identical.

The time of computation was 30 h for GRAPE-1 and 3 h for ETA-10. The former was longer than expected given the nominal speed of 120 Mflops, being in this case limited by the speed of communication because we used a poor combination of our GPIB (general purpose interface bus, IEEE-488) and a personal computer. A better combination, or a VME bus and workstation as planned for later GRAPEs, would have extracted the full speed of GRAPE-1, reducing the computation time by a factor of ten. For computation with $N = 10,000$ the necessary performance of the host is 20 kflops of computation and 50 kbyte s^{-1} of communication, which is satisfied by a personal computer equipped with an Intel 80286 microprocessor for the CPU and 80287 for the numeric accelerator. When such a host machine is used, the fraction of the computation time consumed by the host is $\sim 10^4/(N + 10^4)$, so that the time is divided almost equally between host and GRAPE when $N = 10^4$.

Collisional systems

In globular clusters, gravothermal core collapse proceeds on a timescale of 10^9 yr whereas the binary stars formed in the collapsed core have orbital periods as short as a few months. Despite such a great difference in timescales, the two effects are not readily separable, as perturbation theory would require¹; in other words, the set of differential equations of the type given by equation (1) is quite 'stiff'.

In simulations with a smaller number of particles, the effect of two-body relaxation is overestimated, as discussed above. The difference between these two timescales is therefore less than in real globular clusters, but still too large to be accommodated in simple algorithms.

The program NBODY developed by Aarseth⁸ can handle such problems and is used extensively world-wide. The key to this code is that each particle k has its own time-step Δt_k . The coordinates of k , $\mathbf{x}_k(t_k)$, are stored as a function of its individual time. To advance the time, the minimum value of $t_k + \Delta t_k$ is sought among all the particles; the corresponding particle will be denoted as i . The time t is then advanced to $t = t_i + \Delta t_i$. The

gravitational force is calculated only for particle i ; the positions of the other j particles at t are obtained by extrapolation from $\mathbf{x}_j(t_i)$ and are used to calculate the force on i . Using this force, the host advances the time only for i , that is, the time t_i is replaced with $t + \Delta t$, and the host updates only the data in the memory for i .

This algorithm can easily be incorporated into GRAPE by adding an 'Aarseth prefix' to calculate $\mathbf{x}_j(t + \Delta t)$ by extrapolation. This prefix is placed between the memory for the $\mathbf{x}_k$ s and the first chips for subtractions (Fig. 1). The extrapolation is made by using a polynomial constructed in the pipeline. The Aarseth prefix has its own memory which stores the coefficients of polynomials; these correspond to velocities, accelerations and so on and are sent from the host when it updates the particle positions. Such a machine, which we call GRAPE(A), is now in construction in our laboratory. As it is intended for the study of collisional systems and/or systems with high density contrasts, it is designed to be able to keep 32 or 64 bits of any data within the pipeline. As in GRAPE-1(sw), the force $\mathbf{f}_{ij}$ is calculated in $N + D$ cycle times, but in this case D is several times larger.

GRAPE(A) performs $O(N)$ operations to calculate $\mathbf{f}_i$ for a specific i . The host does only a few operations ($O(1)$) to advance the time for $\mathbf{x}_i$ and to update the coefficients for the extrapolation, and $O(\ln N)$ operations to search for the particle to advance in the next time step. The communications to and from the host are both of $O(1)$. Therefore, the ratio of these loads is not essentially different from that for GRAPE(sw) if we take $O(1) \sim O(\ln N)$. But the real number of operations for N time steps with GRAPE(A) is estimated to be several times that for one time step with GRAPE(sw), so the host in the former case must be several times faster.

Application to other physical systems

Many physical systems can be simulated by integrating the classical equations of motion for many-body systems. We may include short-range forces as well as long-range forces. For instance, hydrodynamic simulations of astrophysical self-gravitating systems are often performed by means of 'smoothed particle hydrodynamics', where the fluid is represented by a system of particles¹¹. The hydrodynamic effects of pressure and viscosity are represented by short-range forces. Molecular dynamics simulations for proteins¹² and for strongly coupled plasmas¹³ are performed by solving the equation of motion for atoms, ions and electrons, taking into account both long-range Coulomb interactions and short-range interactions such as the van der Waals force.

The GRAPE hardware is best suited to the calculation of long-range forces. In particular, the Coulomb force can be calculated without any modification, simply by replacing the particles' mass by their charge.

Short-range forces need be calculated only for pairs of particles lying within the sphere of the force's effective range. Moreover, the form of the force law depends on the problem considered. Therefore, it is better calculated in the programmable host machine. To do this, however, we still have to identify which particles are close neighbours. This involves constructing a list of neighbour particles, which requires a calculation of $O(N^2)$ operations. But the GRAPE hardware determines interparticle distances during the calculation of the long-range forces, and can therefore generate such a list (with only small changes

to the GRAPE hardware) and send it to the host. We call such a machine GRAPE(nl).

A neighbour list can also be useful in the pure N -body problem, by applying Aarseth code with Ahmad-Cohen's neighbour scheme¹⁴ to treat neighbouring particles differently to distant particles. Another application of the neighbour list is in the simulation of an astrophysical system consisting of gas clouds. Each cloud is represented by a 'sticky' particle that exerts a very short-ranged force in addition to the gravitational force.

When the range of the force is long compared with interparticle distances and the number of neighbouring particles is large, the force calculation may be implemented by the GRAPE's hardware logic rather than by the host. There are two ways of calculating a force of arbitrary functional form. The first is to store it in tables. In GRAPE-1 the force is tabulated only for specific values, but it is possible to prepare tables that also include coefficients of interpolation. In this case, the interpolation must be implemented by the hardware logic. Bakker *et al.*^{15,16} have constructed a pipeline in this way to calculate van der Waals' forces for molecular dynamics. The second way is to hardwire the logic specifically to calculate the desired force by using chips such as arithmetic logic units.

Because our GRAPE hardware calculates only forces (and possibly a neighbour list), it is relatively easy to modify the hardware logic as suggested above. In contrast, Bakker *et al.*¹⁵ took a considerable time to construct their molecular dynamics machine because the relevant technology was not then fully developed and because they devised the machine to perform many functions other than a simple force calculation.

Parallel architectures

The force in equation (1) is additive, so it is easy to arrange p pipelines in parallel; we call such a machine GRAPE(p). The N particles are divided into p groups, each group being treated by a separate pipeline. The coordinates $\mathbf{x}_i$ are sent from the host either to a common memory or to memories assigned to each pipeline. The coordinates $\mathbf{x}_i$ of the particle under consideration are sent from the host to all pipelines. Each pipeline calculates the gravitational force from its corresponding group of particles, and the results from respective pipelines are summed at the outlet. The host communicates with the GRAPE pipelines only at the beginning and end of the calculation.

An alternative form of parallelization sets each pipeline to act as if they were single-pipeline GRAPE machines, so that the p pipelines calculate the forces exerted on p particles in parallel. The speed of such a parallel-pipeline system as a whole is almost exactly p times that of the single pipeline. The number of computations to be performed by the host increases linearly with p , whereas operations in GRAPE(p) increase as p^2 . Therefore, GRAPE(p) is appropriate for simulations with very large N .

These logical structures can be combined to produce, say, GRAPE(p, A, nl). By incorporating one pipeline onto a customized LSI chip, arranging one thousand such chips in parallel and clocking them at 30 MHz, a 1-teraflops GRAPE(p, A, nl) is within the reach of present-day technology. The speeds required of the host machine and the interface to drive such a machine are, respectively, 1 Mflops and 1 Mbyte s⁻¹ for $N = 10^6$, and 10 Mflops and 10 Mbyte s⁻¹ for $N = 10^5$. Such speeds are available from present-day workstations. □

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The three-dimensional structure of the bacterial virus MS2

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The structure of the icosahedral bacteriophage MS2 has been determined to 3.3 Å resolution by X-ray crystallography. The phase determination involved both molecular replacement at low resolution using a known structure and heavy-atom substitution. The coat protein has no structural similarity to that of any other known RNA virus.

THE RNA bacteriophages are the simplest phages and so constitute a convenient model system for observing biological events such as viral absorption and penetration, replication and translation of the viral genome, assembly, and viral release¹. They are classified into four major groups according to their serological properties²; MS2 bacteriophage is a member of group I. Its entire genome has been sequenced^{3–6} and is a positive-sense single-stranded RNA molecule of 3,569 nucleotides. The icosahedral protein shell with triangulation number $T=3$ is composed of 180 copies of the coat protein (relative molecular mass (M_r) 13,700); the virion also contains one copy of a maturation protein (the A-protein; M_r 44,000)^{1,7,8}. The coat protein of these bacteriophages serves to protect the viral RNA and acts as a translational repressor of the phage-encoded replicase gene⁹. MS2 infects only male *Escherichia coli* bacteria by injection of its RNA and A-protein^{10,11}. There is some evidence that the A-protein is responsible for the attachment to the bacterial pili and therefore must be exposed on the phage surface¹².

Studies of assembly show that coat protein and RNA form two different complexes, depending on the protein concentration and the protein-to-RNA ratio¹³. Complex 1 is formed at low protein-to-RNA ratios and consists of a coat protein dimer tightly bound to a 21-nucleotide hairpin loop^{14–18}, which is the region on the RNA where the replicase translational initiation site is located. Binding of the coat protein to this region results in a complex that probably corresponds to the translational repressor complex *in vivo*. This complex could also serve as a nucleation site for bacteriophage assembly^{19–21}. Addition of coat proteins to complex 1 results in the formation of complex 2, which is a bacteriophage-like particle composed of one RNA molecule and about 180 coat protein subunits. The absence of the A-protein gives 'defective' particles in which the RNA is partially exposed at one or more sites, making them sensitive to ribonucleases²².

Structure determination

The propagation and purification of MS2 and the crystallization of the virus particle have been described²³. The crystals diffract to at least 2.6 Å resolution. Molecular replacement with a known structure at low resolution, followed by phase extension using non-crystallographic symmetry, have solved the structures of viruses such as mengo²⁴, foot-and-mouth disease²⁵ and bean-pod mottle virus²⁶. Structures of several icosahedral RNA viruses from plant, animal and human hosts²⁷ all have a common structural motif in the individual capsid proteins, which form an eight-stranded antiparallel β -sandwich of identical topology. Although there is no sequence similarity between these proteins and the MS2 coat protein, we expected the MS2 coat protein

also to consist of a β -sandwich, and that these sandwiches would pack as in other $T=3$ viruses. A model of the MS2 protein shell based on the structure of the southern bean mosaic virus²⁸ (SBMV) was used to calculate phases at 13 Å resolution, and the resolution extended stepwise to 3.4 Å. The final R factor and correlation coefficient were 0.204 and 0.890 respectively (Fig. 1). Compared with the corresponding figures from other phase extension calculations, this result seemed quite reasonable, but it was not possible to interpret the map at 3.4 Å.

We assumed that the phases obtained by phase refinement and extension from the SBMV model were partly correct and could be used to locate positions in heavy-atom derivatives. Two derivative data sets (Table 1) gave difference Fourier maps with no positive sites. Instead, some negative peaks were found at positions agreeing with a symmetry of the $T=3$ type. We presumed that these peaks represented normal heavy-atom substitution and were negative because of systematic errors in the phases. These heavy-atom sites were then used for the phase determination and a map calculated at 8.8 Å resolution after isomorphous replacement: phases were extended to 3.3 Å resolution. The total R factor was 0.157 and the correlation, 0.921 (Fig. 1). At 4.0 Å resolution we could trace the main chain in the map. In the final map at 3.3 Å, almost all side chains were visible. A portion of the electron density map is shown in Fig. 2.

Comparing the phases from the first, non-interpretable map with those from this final map, we found that most of them differed by ~ 180 degrees (K. V. *et al.*, manuscript in preparation). The first map was thus the negative image of the subunits. This explains why the difference Fourier map of the heavy-atom derivatives, which were based on these phases, showed negative peaks. It is not clear why the SBMV model gave reasonably correct phases but incorrect signs for the MS2 structure factors. The fact that most of the phases converged to give the Babinet opposite of the structure may be due to the dissimilarity of the two structures and to the absence of most of the low-resolution data at the initial phasing (manuscript in preparation); results

TABLE 1 Data used for structure determination

Resolution range (Å)	Native No. unique reflections	K ₂ PtCl ₄ (%)	No. unique reflections	KAu(CN) ₂ (%)	No. unique reflections	(%)
40.0–20.4	1,485	81.7	433	23.8	220	12.1
20.4–15.8	2,120	95.1	748	33.6	370	16.6
15.8–10.2	11,372	97.1	4,020	34.3	2,119	18.1
10.2–7.5	22,906	97.3	7,490	31.8	4,394	18.7
7.5–5.0	90,574	95.7	12,778	13.5	10,505	11.1
5.0–3.3	280,316	86.5	—	—	—	—
3.3–3.0	98,645	64.4	—	—	—	—
3.0–2.8	23,104	29.8	—	—	—	—
Total 40.0–2.8	540,522	74.6	—	—	—	—
Total 40.0–5.0	—	—	25,469	19.8	17,608	13.7

Oscillation films were processed using the program OSC (refs 43, 44). CCP4 programs (Daresbury, UK) were used for scaling and post-refinement of native data. The final dataset included fully recorded reflections, reflections obtained by adding partial reflections from two adjacent films and reflections obtained by scaling up partials with an estimated partiality of more than 80%. The R value for all reflections was 18.2%. Derivative data sets were obtained by soaking crystals in 5 mM K₂PtCl₄ and 10 mM KAu(CN)₂ respectively.

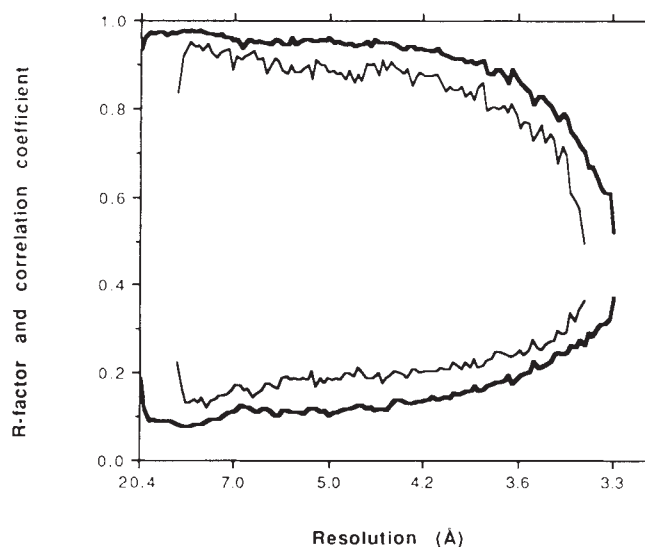


FIG. 1 Statistics for the two final phase refinement cycles. The initial phase extension based on molecular replacement at 13 Å is shown with the final phase extension calculation from isomorphous replacement at 8.8 Å (bold line). The upper two curves show the correlation coefficient, the lower two curves, the *R* factor.

METHODS. The MS2 crystals are monoclinic with dimensions $a=465.9$ Å, $b=287.8$ Å, $c=274.0$ Å and $\beta=121.7^\circ$ and belong to the space group C2. There are two particles in the monoclinic cell with one twofold axis of the virus particle coinciding with the twofold crystallographic axis²³. Thus, the crystallographic asymmetric unit contains one-half of the virion and the icosahedral symmetry gives in this case 30-fold noncrystallographic symmetry. Three-dimensional X-ray diffraction data for native crystals were collected by oscillation photography. Most of the data were recorded on the Wiggler station 9.6 at the SERC Synchrotron Radiation Source (Daresbury, UK). Each high-resolution film covered an oscillation range of 0.5° . The final data set from native MS2 crystals consisted of 424 film packs taken from 103 crystals. Statistics for the native data are shown in Table 1. A self-rotation function calculation³⁹ gave the virus particle orientation. These calculations showed that the virus particle was packed into the crystal lattice in such a way that one of the icosahedral twofold axes coincides with the *a* axis, and that fivefold axes coincide with the *c* axis and the *ab* diagonal. The angle between the *a* and *c* axes is 121.7° and the angle between the *ab* diagonal and the *a* axis is 31.7° ; both angles are very close to the corresponding angles in an icosahedron. A low-resolution model of the MS2 protein shell was constructed from the known SBMV structure. Loops and helices in the three independent SBMV subunits were removed to produce a β -sandwich of 90 amino acids. To account for the smaller radius of MS2, the particle was 'shrunk' by moving pentamers and hexamers of the model subunits radially 18 Å along the fivefold and the threefold axis. Model phases were then calculated and applied to the MS2 data. An initial electron density map was calculated using reflections between 300 Å and 13 Å. F_{obs} had only been measured beyond 35 Å. Calculated structure factors were used where data were missing. The initial map was then averaged 30-fold and the phases cyclically refined⁴⁰. The particle envelope was defined as a shell, 45 Å thick, with an outer radius of 137 Å, which corresponds to the maximal radius along the fivefold axis. After convergence, the phases were extended stepwise from 13 Å to 3.4 Å. In each extension step, new reflections at a slightly higher resolution (less than 1.5 reciprocal lattice points)⁴¹ were added and the phases were refined several cycles by symmetry averaging, using the 30-fold noncrystallographic symmetry. Normally the refinement had converged after five cycles of averaging. At the stage of 4.2 Å resolution the particle envelope was more strictly defined⁴². A new electron density map was calculated with 24,316 independent reflections (33% of all reflections) from 20 to 6 Å resolution using phases obtained from double isomorphous replacement. As only partial data sets of the two derivatives had been collected, they had almost no reflections in common and most of the phases were therefore based on only one derivative. This map was then averaged by the 30-fold noncrystallographic symmetry. After convergence the statistics were poor at resolutions higher than 8.8 Å. These phases were therefore used only to 8.8 Å, and new phases to 3.3 Å resolution were determined by phase extension. During this phase extension, the step size was smaller (0.45 reciprocal lattice points per extension) and the envelope was improved to follow the outer surface of the virus more closely.

are comparable to those obtained for mengo virus after *ab initio* phasing (M. G. Rossmann *et al.*, personal communication).

Monomer structure

The MS2 protein shell is composed of 60 identical triangular units related by the symmetry elements of an icosahedron (Fig. 3*a*). Each triangular unit consists of three identical polypeptide chains (denoted as A, B and C) arranged with roughly threefold symmetry (Fig. 3*b*). The monomer has the shape of a triangle with a base of about 60 Å and a height of 20 Å. The thickness

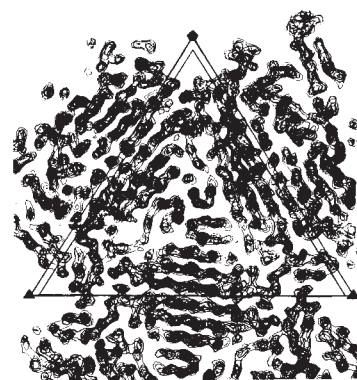


FIG. 2 Sections from 109 Å to 116 Å of the 3.3 Å averaged electron density map, cut perpendicular to one icosahedral twofold axis. The triangular wedge defines the icosahedral asymmetric unit as described in Fig. 3.

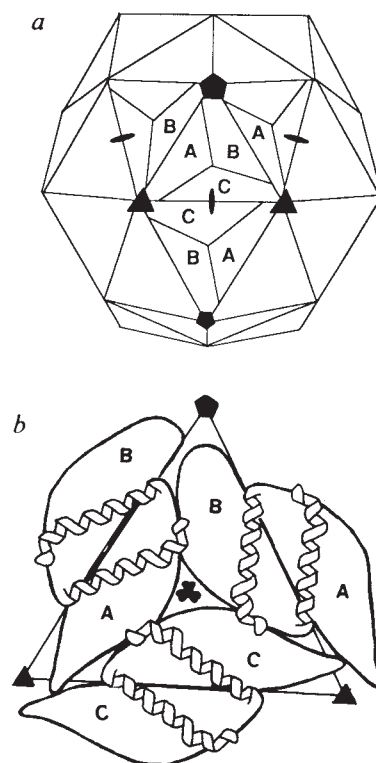


FIG. 3 *a*, Icosahedron with all symmetry elements of one triangular face indicated. An icosahedral symmetric unit is defined as the triangular region between two threefold and one fivefold axis. *b*, Positions of the subunits of MS2 in the icosahedral symmetric unit and neighbouring subunits in the capsid.

of the shell is 20 Å. The tertiary structure and topology of the MS2 coat protein is unlike that of other icosahedral viruses. The main chain of the protein subunit folds into seven β -strands and two α -helices (Fig. 4). Five of the β -strands (β C, β D, β E, β F and β G; residues 21–24, 30–34, 42–51, 56–66 and 82–94) make up a twisted sheet of the β -meander type and form the RNA-facing side of the subunit. The remaining two antiparallel strands (β A and β B, residues 7–10 and 18–20), which are on the outside of the particle, cross the β C– β G sheet at an angle of about -35° . The short loop between strands A and B is not as well defined in the electron density map as the rest of the protein. It is probably slightly flexible owing to its sequence (-Gly-Gly-Thr-Gly-), and it is exposed on the outer surface of the virion. The two C-terminal helices, α A and α B, protrude from the core of the subunit like a tail. Their exact position in relation to the rest of the subunit is probably determined by the dimer interaction. Helix α A (residues 97–111) is almost parallel to strands β A and β B. The structure ends with a short helix, α B (residues 117–125). Many amino acids of the subunit are involved in a well defined secondary structure ($\sim 50\%$ β -sheet and $\sim 20\%$ α -helix) stabilized by intramolecular hydrogen bonding.

The fold of the three independent subunits is essentially identical, except for the long loop between β F and β G (residues 67–81). In subunits A and C, the two strands β F and β G are extended and there are five additional hydrogen bonds between the strands, but in subunit B the long loop has a completely different conformation. There is also a minor difference in the DE loop between subunits B and C (residues 35–41). If the FG loop is omitted, the r.m.s. distance of the α -carbon positions after a superposition of the subunits is 0.7 Å (A on C) and 0.8 Å (A on B).

Some density less than the density of the ordered protein side chain is found at certain positions just below the protein shell, most notably extending from the residues Thr 45 of subunits A and C. This density is close to the side chains of Lys 43 and Lys 61. It seems possible that this density could represent parts of the RNA molecule. Apart from this weak and mostly discontinuous density, the RNA molecule and the A-protein are not seen in the electron density map. This implies that these molecules are disordered with respect to the protein shell, probably as a result of random packing of the icosahedral particle in the crystal.

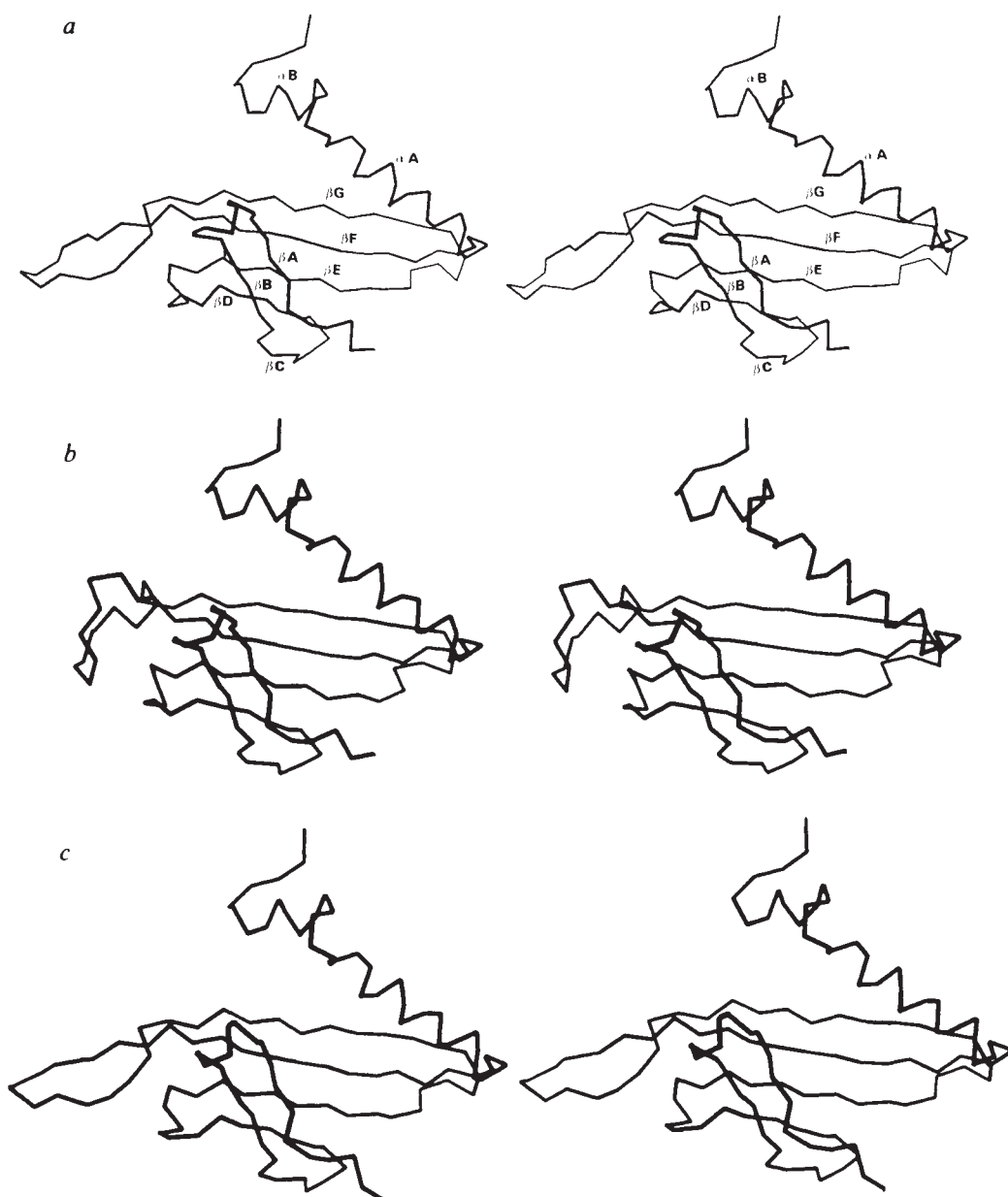


FIG. 4 Stereo diagrams of α -carbon models showing the polypeptide chain fold of the three MS2 subunits in the icosahedral asymmetric unit as viewed from the outside of the virus particle. *a*, A subunit; *b*, B subunit; *c*, C subunit. A complete model of the three independent coat protein subunits was built using the program O (developed by Alwyn Jones and coworkers) and an Evans and Sutherland PS330/PS390 graphics system.

Dimer structure

In an icosahedral $T=3$ structure, there will be two types of dimer arrangements: C/C dimers at a twofold axis and A/B dimers at a quasi-twofold axis (Fig. 5a). The two dimers were superimposed (Fig. 5b). They are similar, and the r.m.s. distance of the α -carbon positions, excluding the FG loop, is 0.8 Å. The contact surface in the MS2 dimer is unusually extensive. First, the long β G strands of the two interacting subunits form eight hydrogen bonds, thus extending the β -sheet of one subunit into a 10-stranded sheet in the dimer. Second, the C-terminal helical tail from one subunit is situated in a groove in the other subunit, which is formed between the β A/ β B hairpin and the corresponding helical tail of that subunit (Fig. 5c). In this way the N terminus from one subunit interacts with the C terminus of the other subunit in the dimer. The interaction within the dimers is mostly hydrophobic. The helices have a hydrophobic surface, consisting of Cys 101, Ile 104, Met 108, Leu 111, Leu 112, Pro 117, Ile 118 and Ile 122, that is in contact with Leu 90 and Ile 92 in the β -sheet of the same subunit and Val 64, Leu 86, Ile 60, Met 88, Met 108, Cys 46, Phe 7, Tyr 58 and Leu 90 from the other subunit in the dimer. As evidenced by the extensive hydrophobic interaction and the similarity between the structurally independent dimers, the dimer is compact and probably relatively rigid, except for the 15-residue FG loop; it could be the building block for shell formation.

Other subunit interactions

The subunit contacts close to the quasi-threefold axis are formed by segments of strand β C, the loop between β C and β D and by the C terminus. The subunits are located at slightly different radii, with subunit C at the smallest and B at the largest, about 5 Å above the C subunit in the CD loop. This means that the interactions between subunits around the quasi-threefold axis

are not identical. The FG loop is responsible for the interaction close to the threefold and fivefold axes (Fig. 6). At the threefold axis the similar and extended loops of the A and C subunits form a roughly sixfold contact, leaving a hole of ~ 17 Å diameter. At the fivefold axis, the FG loop of the B subunit is bent towards the rest of the subunits. This results in a cylindrical channel which is ~ 14 Å in length and 16 Å in diameter. In contrast to the contacts within the dimer, most of the interactions between the dimers and the FG loops are mostly hydrophilic.

The quaternary structure of the MS2 capsid gives the virus an almost spherical shape. The surface consists of α A/ α B helices and β A/ β B hairpins. The protein shell seems to be relatively porous, with large channels at the five- and threefold axes. The inside of the protein shell is hydrophilic and exposes several clusters of positively charged residues (Arg 38, Lys 43, Arg 49, Lys 57, Lys 61 and Arg 83 on the three independent subunits). The side chains of some of these residues are partly disordered and are probably involved in interactions with the RNA.

Structural comparisons

There is a similarity in the topology and tertiary structure of the coat proteins of all icosahedral plant, insect and mammalian RNA viruses studied so far²⁷ and this extends to the hexon protein of adenovirus, which is a large icosahedral DNA virus²⁹. The only other example of an icosahedral shell built up of proteins with structures not having the β -sandwich motif is found in a bifunctional enzyme complex, heavy riboflavin synthase³⁰. This complex consists of 60 identical β -subunits, which form an icosahedral $T=1$ capsid enclosing three α -subunits in the central core. The main chain of the β -subunit folds into a parallel four-stranded β -sheet structure flanked on both sides by two α -helical segments.

The main-chain folding of the MS2 dimer is not unlike that in the bovine platelet factor 4 dimer³¹ and in one domain of the class I histocompatibility antigen, HLA-A2 (ref. 32). Both structures are composed of a β -sheet, six- or eight-stranded, and

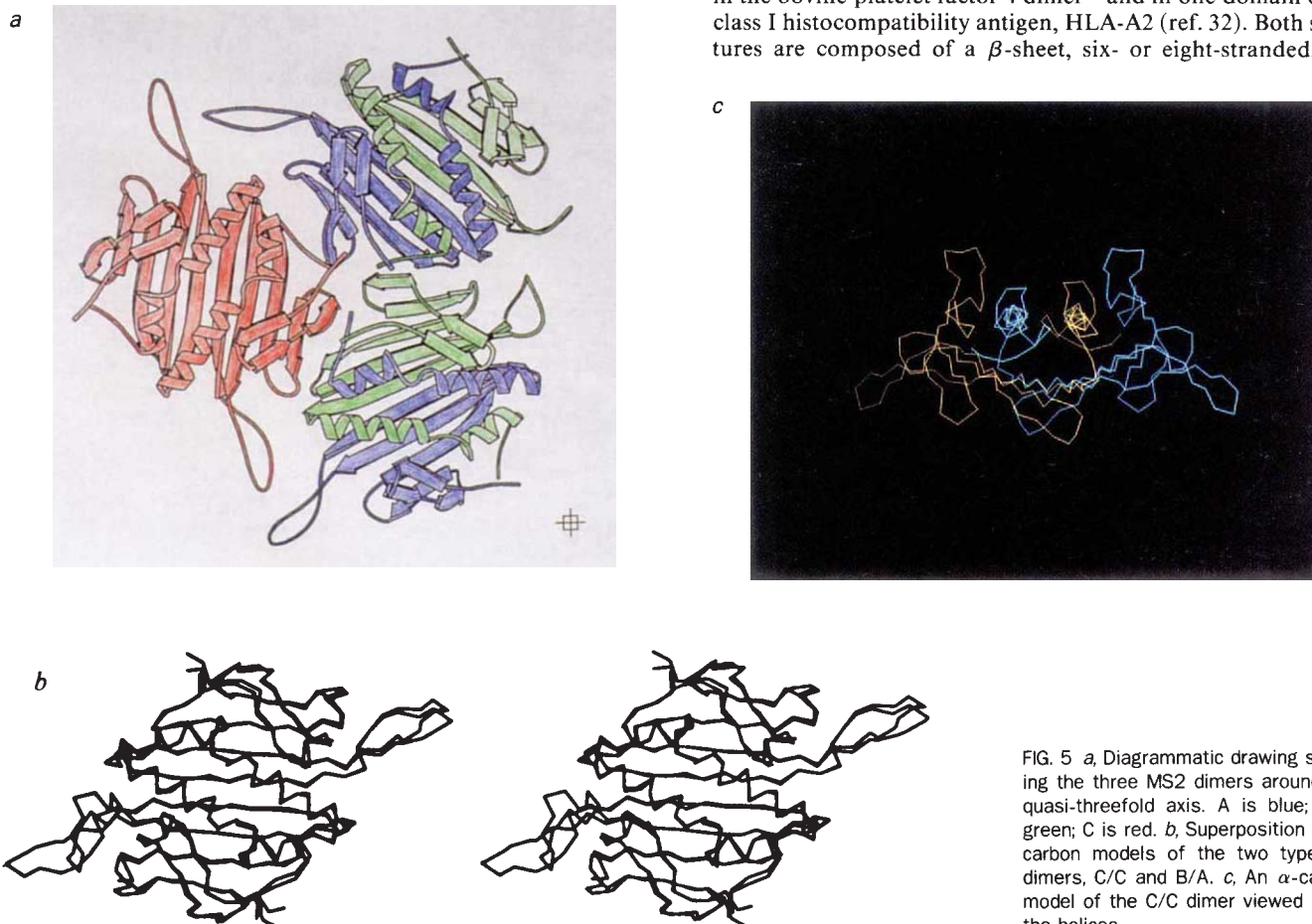


FIG. 5 a, Diagrammatic drawing showing the three MS2 dimers around the quasi-threefold axis. A is blue; B is green; C is red. b, Superposition of α -carbon models of the two types of dimers, C/C and B/A. c, An α -carbon model of the C/C dimer viewed along the helices.

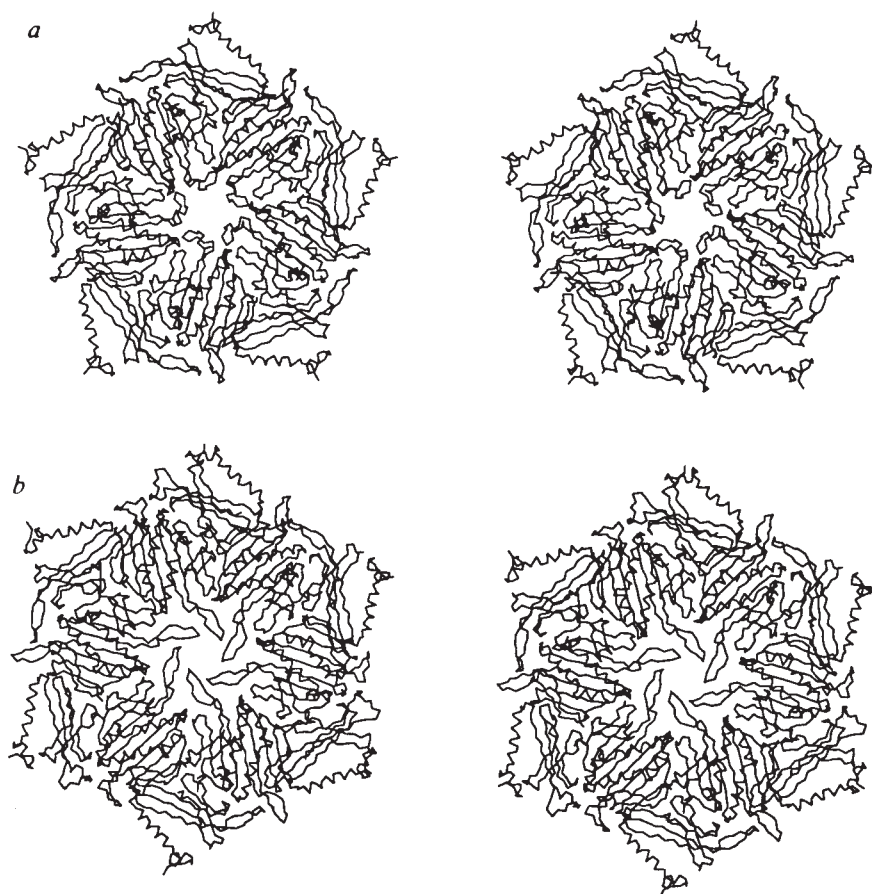


FIG. 6 Stereo diagrams showing α -carbon models as viewed down *a*, a fivefold axis and *b*, a threefold axis from the outside of the virus particle.

have two antiparallel helices on one side of the β -sheet. The angle between the helical axis and the strands differs between these structures: it is $+52^\circ$ in platelet factor 4 dimers and -45° in HLA-A2, compared with -35° in MS2 coat protein. Another difference is that these two proteins have a 10–14 Å-wide groove between the two helices.

The coat protein sequence is known for several small RNA phages representing all four serological groups of *E. coli* phages, as well as two *Pseudomonas* bacteriophages. Optimal alignment of these sequences shows that only a few residues are invariant. The greatest similarity is in strands βE , βF and βG : Pro 78 in the FG loop is conserved in all sequences and so is Thr 45 and Ser 47 in strand βE . Why these residues should be conserved is not obvious, but the electron density found close to Thr 45 indicates that it could be involved in interactions with the RNA molecule.

Assembly

Models for the assembly of $T=3$ viruses have been proposed on the basis of the structure of some plant and insect viruses^{33,34}. In a $T=3$ virus, the chemically identical subunits have to adapt to structurally different environments, causing problems for assembly. The assembly of all $T=3$ viruses studied so far is regulated by a folded arm present at some of the equivalent subunit interfaces. This arm, which is ordered in one of the subunits, is like a switch, determining the type of contact and the local curvature of the shell; it is essential for specification of the $T=3$ structure, because protein subunits form $T=1$ particles if the arm is removed^{33,35}. The MS2 coat protein can self-assemble into virus-like shells of the same size as the complete virus^{36,37}, so the coat protein is able to regulate correct assembly into a $T=3$ particle. The interaction of subunits in MS2 is different from other $T=3$ viruses because there are no

arms to regulate dimer interaction at assembly. Instead the dimer is relatively rigid. Evidently the different dimer-dimer contacts must be sufficiently specific to control the assembly mechanism.

It has been shown that the coat protein binds as a dimer to the 21-nucleotide hairpin loop, the replicase translational operator^{16,17}. The RNA is not needed for assembly, but the presence of the complete RNA molecule or even of only this hairpin loop stimulates the coat protein polymerization³⁸. At present, we are not able to propose a possible pathway for the assembly or suggest where the A-protein is located. We do not consider it likely that one dimer could be replaced by the A-protein because this would disturb the symmetry of the viral shell.

Establishment of the MS2 coat protein subunit structure will give new insight into the structure and assembly of small spherical viruses. As more becomes known about the three-dimensional structure of MS2, it will supplement information on protein stability, assembly and RNA recognition obtained with MS2 coat protein mutants. □

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A binding site for the T-cell co-receptor CD8 on the α_3 domain of HLA-A2

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Adhesion measurements between CD8 and 48 point mutants of HLA-A2.1 show that the CD8 α -chain binds to the α_3 domain of HLA-A2.1. Three clusters of α_3 residues contribute to the binding, with an exposed, negatively charged loop (residues 223–229) playing a dominant role. CD8 binding correlates with cytotoxic T-cell recognition and sensitivity to inhibition by anti-CD8 antibodies. Impaired alloreactive T-cell recognition of an HLA-A2.1 mutant with reduced affinity for CD8 is not restored by functional CD8 binding sites on an antigenically irrelevant class I molecule. Therefore, complexes of CD8 and the T-cell receptor bound to the same class I major histocompatibility complex molecule seem to be necessary for T-cell activation.

MAJOR histocompatibility complex (MHC) glycoproteins bind peptide antigens and present them to T lymphocytes (reviewed in ref. 1). Class I and II MHC molecules respectively present antigens to T cells expressing the CD8 and CD4 glycoproteins^{2,3}. These correlations result from specific binding of class I molecules by CD8 and of class II molecules by CD4 (refs 4–8). CD4 and CD8 are thought to function as antigen non-specific accessory molecules, serving to augment the affinity of antigen-specific T-cell receptors (TCR). CD4 and CD8 are also implicated in transmembrane signalling and the regulation of T-cell proliferation. For example, anti-CD4 and anti-CD8 antibodies can inhibit lectin-mediated or MHC non-specific activation or

lysis of target cells^{9–13}, or synergize with anti-CD3 antibodies (which trigger the antigen specific TCR) to deliver a potent mitogenic signal^{14–16}. These latter results imply that CD4 and CD8 act as co-receptors, required in combination with specific TCRs to give efficient antigen recognition, a concept consistent with association of a protein tyrosine kinase (p56^{lck}) with the intracellular domains of CD4 and CD8^{17–21}. In addition to their role in antigen recognition by mature peripheral T cells, CD4 and CD8 are key elements of T-cell selection in the thymus^{22–25}.

In co-receptor models²⁶, simultaneous binding of antigen-specific TCR and the appropriate co-receptor (CD4 or CD8) to the same complex of MHC molecule and peptide antigen is important for T-cell activation. Such models therefore predict that the CD8 and TCR-binding sites of the class I MHC molecule do not overlap. Interpretation of studies on T-cell antigen recognition, in terms of the crystallographic structures of two human class I MHC molecules, HLA-A2.1 and HLA-Aw68.1, indicate that the TCR interacts with a surface formed by the upper faces of the helices of the α_1 and α_2 domains and exposed parts of the peptide sandwiched between them^{27–29}. By contrast, characterization of mutant murine and human class I molecules, substituted at positions 227 and 245, point to the α_3 domain being a site of contact with CD8 (refs 30–33). We now report experiments that substantiate this hypothesis, map the binding site, correlate changes in CD8 binding with T-cell recognition and provide evidence supporting the co-receptor model for CD8 function.

Clusters of α_3 residues affect CD8 binding

A panel of 48 mutant HLA-A2.1 genes, each differing from the wild type by a single amino acid substitution, were transfected into the HLA-A,B-negative B cell line CIR (Fig. 1a). Mutations were mostly made in the α_3 domain at positions surrounding 227 and 245; although more distant positions, selected to be at the surface of α_3 , were also included. To examine the possibility that CD8, like TCR, binds to the helices of the α_1 and α_2 domains³⁴, mutations were also made in conserved residues on

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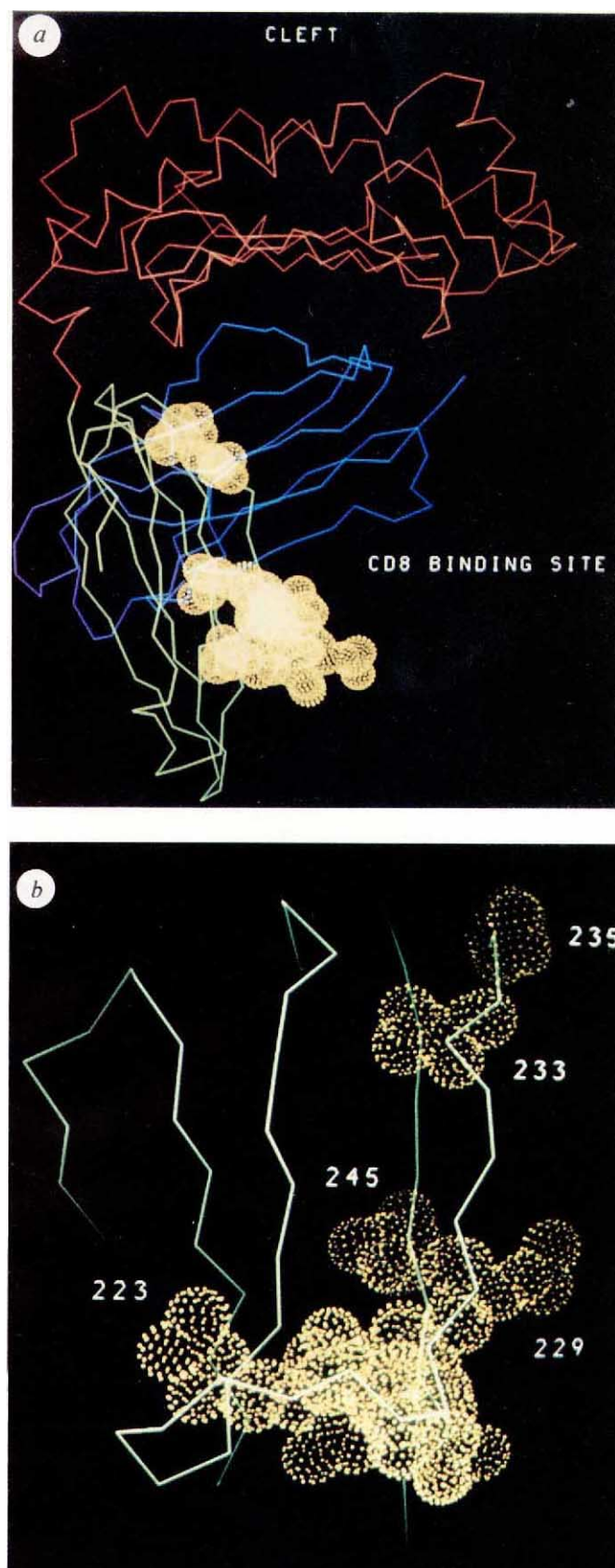


FIG. 2 CD8 binding residues of the α_3 domain. *a*, View of the HLA-Aw68.1 molecule perpendicular to the $\alpha_1 + \alpha_2$ helices²⁹. Alpha carbon backbone for the $\alpha_1 + \alpha_2$ domains is in red, for the α_3 domain in green, and for β_2 -m in blue. In yellow are the van der Waal's surfaces of residues in the α_3 domain important for CD8 binding. *b*, Isolated view of the α_3 domain. Individual residues are numbered according to position.

the upper faces of the two helices (Fig. 1*a*). Transfected cells were compared for cell-surface expression of the mutant proteins, for binding to CD8 in cell-cell adhesion and for recognition by specific alloreactive cytotoxic T cells (CTL).

Reduced binding in the cellular adhesion assay can result either from reduced affinity for CD8 or from insufficient cell-surface expression of a mutant HLA-A2.1 molecule. Cell-surface expression was monitored in every assay and a reproducible loss of adhesion was only attributed to a change in the CD8-binding site for transfectants with levels of expression greater or equal to at least one of the CD8 binders. This criterion was designed to prevent artefactual assignment of residues to the CD8-binding site, and in consequence the analysis will not score mutations that simultaneously result in a significant loss of cell surface expression and a reduced affinity for CD8. Therefore this study results in description of a 'minimal CD8-binding site'.

Thirty-nine of the mutant proteins gave informative results in the adhesion assay, being expressed on the cell-surface at levels $\geq 40\%$ of wild type A2.1 (Fig. 1*b*). Of these, 25 bound to CHO cells expressing the human CD8 α -chain, including all 4 molecules with mutations in either the α_1 or α_2 helices (Fig. 1*c*). Only substitutions in the α_3 domain caused reduced interaction with CD8 and they fall into three separate clusters within the primary sequence. The first cluster includes positions 223–229, which form a highly exposed loop between strands 3 and 4 of the α_3 domain (Fig. 2). The sequence from positions 220–232, which includes the loop, contains 6 acidic residues (220, 222, 223, 227, 229 and 232), no basic residues and is conserved in 76 out of 77 HLA-A,B,C heavy chain sequences³⁵. The accessibility and sequence conservation, combined with the effects of mutation on CD8 binding, argue for direct involvement of the loop residues in contacting the CD8 α -chain and a role for negative charge.

The second cluster involves positions 233 and 235 of the fourth strand of β -pleated sheet and is somewhat apart from clusters I and III. Position 233 is on the exposed surface of the α_3 domain. The fact that relatively conservative changes of threonine 233, to either alanine or isoleucine, eliminate CD8 binding indicate this residue may directly contact CD8. In comparison, residue 235 is substantially buried in the interface between α_3 and β_2 -microglobulin (β_2 -m) and one effect of substitution may be to change the interaction with β_2 -m. Mutation at 235 removes a proline residue which could also affect the main chain conformation of α_3 . A rare HLA-A2 subtype (A*0209) differs from HLA-A2.1 (A*0201) by a substitution of glutamic acid for alanine at 236 (ref. 36), a position close to cluster II. The mutant with this substitution however, showed no significant changes in binding to CD8 in the adhesion assay (Fig. 1*c*).

The third cluster comprises positions 245 and 247, located on the fifth strand of β -pleated sheet behind the cluster I loop residues. Replacement of valine for alanine at position 245 causes loss of CD8 binding by Aw68.1 (ref. 33), and comparison of the crystallographic structures of A2.1 and Aw68.1 shows that although without effect on the conformation of the α_3 domain, this substitution causes a movement in the side chain of the loop residue threonine 228 (ref. 29; T.P.J.G., M. Saper and D. Wiley, unpublished observations). Substitution of glycine for alanine 245 has no effect upon CD8 binding, indicating the size difference of the alanine and valine side chains is the primary cause of the difference in CD8 binding between A2.1 and Aw68.1. This may directly alter contact with CD8, as residue 245 is partly exposed on the surface of the α_3 domain, or its effect could be through the transmitted change in residue 228. That substitution of serine for alanine 245 abrogates CD8 binding also supports side chain size being the critical factor. Loop residues 218–228 protrude from the α_3 domain and are in part supported by residue 247, which is buried. The effects of substitution at position 247 are probably due to small changes in the position or conformation of the loop.

<i>a</i>		
Name	Position	Mutation
37Y	37	D>Y
58V	58	E>V
157T	157	R>T
158H	158	A>H
177K	177	E>K
		α_{1+2}
210S	210	P>S
214A	214	T>A
215A	215	L>A
216A	216	T>A
217A	217	W>A
218A	218	Q>A
219A	219	R>A
220A	220	D>A
221V	221	G>V
221E	221	G>E
222A	222	E>A
223A	223	D>A
224A	224	Q>A
224E	224	Q>E
225A	225	T>A
225D	225	T>D
226A	226	Q>A
227A	227	D>A
227K	227	D>K
228A	228	T>A
228E	228	T>E
229A	229	E>A
230A	230	L>A
230P	230	L>P
231A	231	V>A
232A	232	E>A
233I	233	T>I
233A	233	T>A
234A	234	R>A
235A	235	P>A
236E	236	A>E
239R	239	G>R
242K	242	Q>K
243A	243	K>A
244A	244	W>A
245G	245	A>G
245S	245	A>S
245V	245	A>V
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247A	247	V>A
251P	251	S>P
265V	265	G>V
265A	265	G>A
		α_3

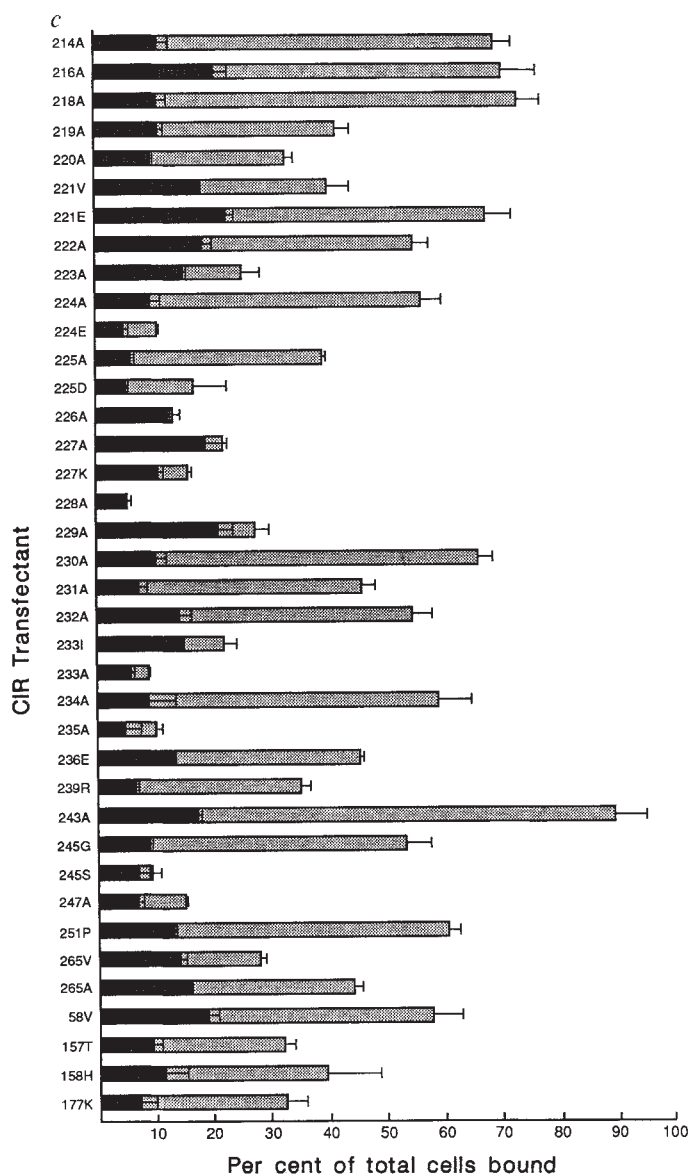
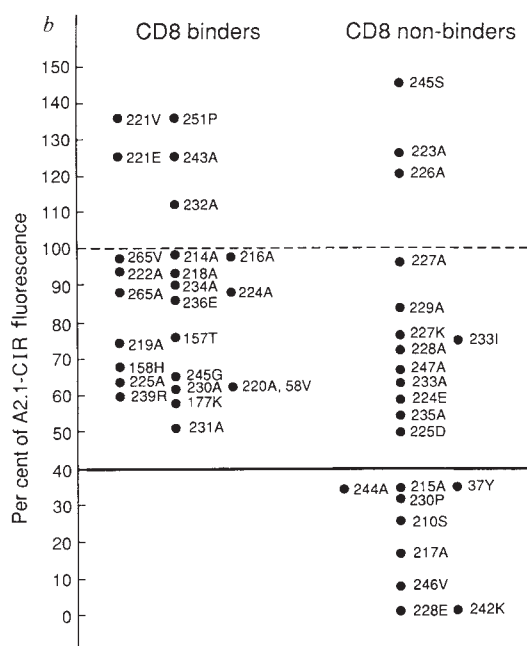


FIG. 1 Mutant HLA-A2.1 binding to CD8. *a*, List of point mutations of HLA-A2.1. *b*, Surface expression of mutant HLA-A2.1 molecules in CIR cells as assessed by flow cytometry. Staining was with CR11-351, an HLA-A2/A28 specific monoclonal antibody⁴¹. Mean fluorescence channel values were normalized to wild-type HLA-A2.1 expression, shown by the horizontal broken line. Results were obtained in three separate experiments performed at the same time as the three binding experiments shown in (*c*). Day-to-day variation in the absolute levels of cell-surface expression was 0–30%. Division of transfectants into CD8 binders and non-binders is based on multiple adhesion assays, examples of which are shown in (*c*). The CD8 non-binders include two groups of mutants: those with mean expression levels $\geq 40\%$ of wild-type (the cut-off is given by the horizontal solid line) which are assigned as having a reduced affinity for CD8 and those with expression levels $< 40\%$ of wild-type which are assigned as having impaired expression (as defined by a panel of monoclonal antibodies) and for which reduced CD8 binding could derive either from the low expression or a loss of affinity for CD8. *c*, Adhesion of CIR cells expressing mutant HLA-A2.1 molecules to CHO cells expressing the transfected CD8 α -chain gene (light stipple) or mock-transfected CHO cells not expressing CD8 α -chain (heavy stipple). Because of the limited number of samples that can be processed in a single assay, the results shown are a composite of three experiments. The adhesion assay was robust and reproducible, provided that cell-surface expression of the mutant molecules, which was monitored in every assay, did not dramatically change. Reproducibility in data and in assignment of transfectants as CD8-binding or non-binding was demonstrated in three ways: for each mutant 2–5 repeated assays with the same transfectants were made and in multiple assays gave similar results; for 19 mutants, independent transfectants were made and gave similar results in multiple assays; 6 mutants (210S, 221E, 221V, 227K, 233I and 242K) were exon shuffled onto molecules having the α_1 and α_2 domains of HLA-Aw68.1 and on transfection and assay gave similar results and assignments as the HLA-A2.1 mutants. METHODS. Mutations in α_1 and α_2 were as described⁴². Oligonucleotide directed mutagenesis of α_3 was as described⁴³ and a 1.5 kilobase *KpnI*/*NcoI* fragment of the HLA-A2.1 gene⁴⁴ encoding α_3 and transmembrane domains was subcloned into M13mp18. All mutations were confirmed by sequencing exon 4. Mutations were inserted into HLA-A2.1 in the plasmid vector pHEBO⁴⁵ by *Bgl*III and *Afl*III digestion and ligation of gel purified fragments. Transfection into CIR cells, flow cytometric analysis of expression and CD8 adhesion assays were as described^{5,33}.

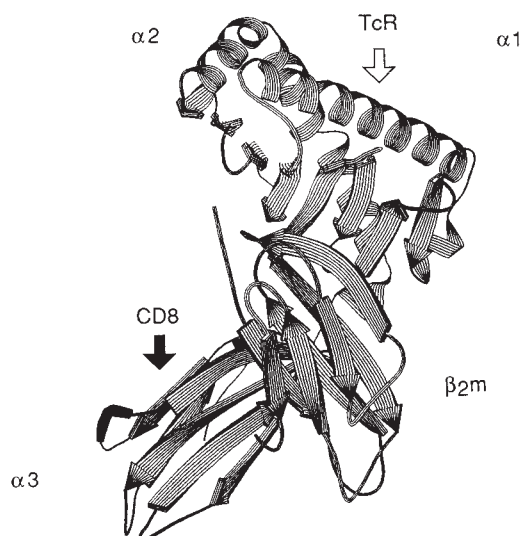


FIG. 3 Accessibility of the CD8 binding site shown on a schematic representation of HLA-A2.1 structure. β strands are shown as thick arrows and α -helices as helical ribbons. The three domains of the heavy chain and β_2 -m subunit are marked. Also indicated are putative TCR and CD8-binding sites in the α_1 - α_2 domains and α_3 domain respectively. The loop connecting strands 3 and 4 of the α_3 domain, which is crucial for CD8 binding, is marked with a thick line. The figure is modified from Fig. 2 of ref. 35 and its use for visualizing the CD8 binding site was suggested by M. Saper.

This analysis shows that the loop between strand 3 and 4 of the α_3 domain is critical for interaction with the CD8 α -chain and probably provides a site of direct contact. The region of contact between CD8 and the α_3 domain may extend beyond the loop and include exposed residues of strand 4 (Fig. 2). This localized area of the α_3 domain forms an exposed surface that can readily be envisaged as providing a contact site for CD8 (Fig. 3). Our results strongly indicate that CD8 and TCR bind to distinct non-overlapping sites of the class I MHC molecule, although this cannot be formally proved until direct assays for binding of the TCR are developed.

CD8 binding correlates with T cell recognition

The conditions under which CD8 binding is examined in the *in vitro* adhesion assay are different from those that a class I MHC molecule would encounter *in vivo*. For example, the CHO transfectants have greater numbers of CD8 molecules than T cells and express only the CD8 α -chain homodimer and not the $\alpha\beta$ heterodimer^{37,38}. It was therefore important to examine the effects of mutations in the α_3 domain of HLA-A2.1 upon recognition by T cells. In these experiments, the lysis of CIR transfectants expressing mutant A2.1 molecules by bulk cultures of A2.1 specific alloreactive cytotoxic T cells (CTL) and by clones of CTL derived from these cultures was assessed.

Significant correlation between the results of the adhesion and cytolytic assays was obtained. The α -3 mutants that retained CD8 binding were unaffected in their recognition by CTL, whereas mutations that resulted in loss of CD8 binding were less effectively recognized by CTL. Differential reductions in cytotoxicity were also observed between the various substitutions that eliminated CD8 binding in the adhesion assay; cluster I substitutions having the greatest effects, cluster II substitutions the least and cluster III substitutions being intermediate. Within the cluster I substitutions, a progressive loss and regain in CTL recognition can be discerned at successive positions through the exposed, negatively charged loop (Fig. 4).

CTL were differentially sensitive to the panel of mutants; bulk cultures showed some lysis of all the mutants, whereas greater discrimination was seen with clones (Fig. 4). Eight CTL clones were analysed in detail and no two gave identical patterns of lysis; they exhibited a range of sensitivity to mutations affecting

CD8 binding indicating differing degrees of CD8 dependence. Lysis by three clones, representing high, intermediate and low sensitivity, is shown in Fig. 4. Clone AMSH10 is relatively CD8-independent as it is not affected by cluster II and III mutations, and only moderately by cluster I mutations. Even in this case T-cell recognition is impaired, as seen by the poor lysis of the 227 lysine mutant. In contrast, clone AL1.1 is dependent on CD8, exhibiting greatly reduced lysis with all cluster I and III mutations. These two clones, representing the extremes of the observed range of CD8 dependence as assessed by cytotoxicity, were tested for inhibition with monoclonal anti-CD8 antibody—the conventional assay for CD8 dependence.

With wild-type A2.1 as the target, an eightfold greater amount of anti-CD8 antibody was required to inhibit clone AMSH10 compared to clone AL1.1. This demonstrates correlation between CD8 dependence as defined either by cytotoxicity of cells expressing α_3 mutations, or by antibody inhibition. We next compared the amounts of anti-CD8 antibody required to inhibit lysis by clone AMSH10 of targets expressing A2.1 molecules with normal and with defective CD8-binding sites. Much less antibody, comparable to that required to inhibit clone AL1.1, was required to inhibit lysis of a cluster I mutant compared with wild type (Fig. 5). Therefore the potency of anti-CD8 antibody to block AMSH10 is not just a function of the T cells but is dependent upon the affinity of CD8 for the target class I molecule. This indicates that anti-CD8 antibody is competing with class I MHC molecules in binding to CD8 and that reduction in strength of the class I-CD8 interaction results in a given antibody being a better competitor.

Co-receptor function for CD8

If CD8 is purely a non-antigen-specific adhesion molecule, then cells expressing a CD8 non-binding mutant A2.1 molecule (not killed by CD8 dependent A2.1-specific CTL) should be rendered susceptible to lysis if a second class I molecule, not recognized by the TCR of the CTL but having a functional CD8 binding site, is introduced. On the other hand, if complexes of TCR and CD8 with the same class I molecule are important, then no increase in susceptibility to lysis would be expected. To distinguish these possibilities a transfectant expressing both a non-CD8 binding mutant of A2.1 and HLA-B7, which does bind CD8, was made. The A2.1 mutant with alanine 245 substituted for valine was chosen because it is the substitution found in Aw68.1, for which the three-dimensional structure is known²⁹. As expected, the transfectant expressing both B7 and the A2.1 mutant bound CD8 in the adhesion assay, whereas the transfectant expressing just the A2.1 mutant did not. However, lysis of the A2.1 mutant by the CD8 dependent clone AL1.1 was not improved by the simultaneous expression of B7 (Fig. 6a, b).

To address the possibility that the α_3 mutation at position 245 affected recognition of α_1 and α_2 by the TCR, although no structural change is predicted from a comparison of the three-dimensional structures of HLA-A2.1 and Aw68.1, the capacity of the mutant A2.1 molecule to act as a cold-target inhibitor was measured. Transfectants expressing the mutant A2.1 molecule, but not those expressing an irrelevant class I molecule (HLA-B27), were able to inhibit the lysis of A2.1 expressing cells by clone AL1.1, showing that the mutant molecule was in fact recognized by TCR of this clone even though no lysis was evident (Fig. 6c, d). The inhibition by the mutant was not as potent as that seen with A2.1, suggesting that the avidity of the cellular interaction is lowered by the CD8 binding deficiency.

These results support a co-receptor model in which interactions between CD8 and the same class I peptide complex that is engaging TCR are essential for cytotoxicity by the T cells studied here. A role for non-specific CD8-class I adhesion in strengthening T-cell target interactions is not brought into question by our findings. They do, however, indicate that such interactions are not sufficient to fully explain CD8 function; a view supported by the finding that irrelevant class I molecules can enhance but

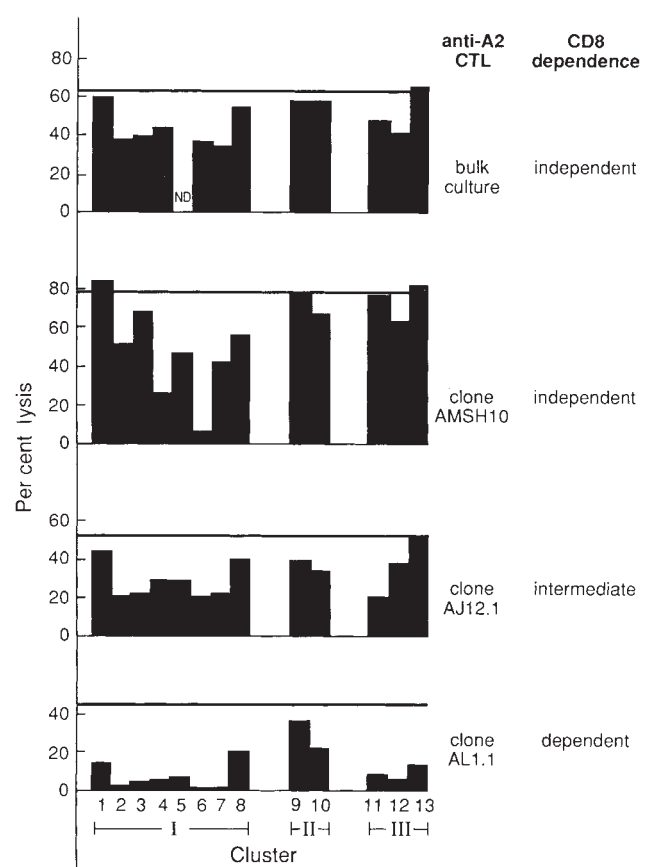


FIG. 4 Heterogeneity in CTL recognition of targets expressing CD8 non-binding mutants of HLA-A2.1. Lysis of targets by an HLA-A2 allo-specific bulk T-cell culture is shown in the top panel. Target cells are those that do not bind CD8 in the adhesion assay and are (1) 223A, (2) 224E, (3) 225D, (4) 226A, (5) 227A, (6) 227K, (7) 228A, (8) 229A, (9) 233I, (10) 235A, (11) 245V, (12) 245S, (13) 247A. Lysis of HLA-A2.1-CIR and untransfected CIR cells were 61% and 0% respectively at an effector:target ratio (E:T) of one. All CD8 binding mutants were lysed strongly by this culture. Lysis of the same targets by three HLA-A2 allo-specific CTL clones is also shown. Eleven clones were initially screened on a limited group of targets. Of these, eight were further tested on the targets shown. Three representative clones exhibiting high, medium and low sensitivity to the mutations are shown. The other five clones each exhibited a pattern of lysis similar to one of these three. Clone AMSH10 was used at an E:T of 2 and lysed HLA-A2.1 CIR and CIR cells 77% and 6% respectively. Clones AJ12.1 and AL1.1 were used at an E:T of 1. HLA-A2.1-CIR and CIR targets were lysed 51% and 1% (AJ12.1) and 44% and 0% (AL1.1) respectively. In each panel, a horizontal line indicates lysis of A2.1-CIR targets.

METHODS. PBL from a normal donor (HLA-A3, B7, 38, DR6) were stimulated repeatedly with the HLA-A2.1-expressing cell line JY to generate a CD8-positive HLA-A2 allo-specific culture AJY⁴⁶. PBL from the same donor were stimulated first in bulk with several different HLA-A2 bearing cells, then cloned by limiting dilution on irradiated feeder layers of HLA-A2 cells in the presence of T-cell growth factors, including IL-2. Clones obtained were CD8-positive and cytotoxic for HLA-A2 bearing targets. No reactivity with non-HLA-A2 targets was observed. CTL were assayed for cytotoxic activity by 4-h ⁵¹Cr release assay as described. Per cent lysis = experimental release - spontaneous release ÷ total release - spontaneous release.

FIG. 5 Differential inhibition of cytotoxicity by anti-CD8 antibody. Lysis of CIR transfectants (E:T=5) was inhibited by anti-CD8 antibody, Campath 8c (■), and by anti-class I specific antibody, W6/32 (●), but not by anti-clathrin light chain-specific antibody CVC.7 (▲). To achieve 50% inhibition of lysis, the concentration of Campath 8c antibody required was 7.5 $\mu\text{g ml}^{-1}$ for AMSH10 lysis of A2.1-CIR, 0.6 $\mu\text{g ml}^{-1}$ for AMSH10 lysis of 224E-CIR, and 1.0 $\mu\text{g ml}^{-1}$ for AL1.1 lysis of A2.1 CIR. Lysis of 224E-CIR by AL1.1 was 16% in the absence of antibody. Untransfected CIR cells gave 9% specific lysis.

METHODS. Campath 8c is a rat-derived monoclonal antibody obtained from Dr Herman Waldmann. W6/32 and CVC.7 are mouse-derived monoclonal antibodies described previously^{47,48}. Each was preincubated for 30 min at room temperature with CTL before addition of ⁵¹Cr-labelled targets. The assay was then continued as described in Fig. 4.

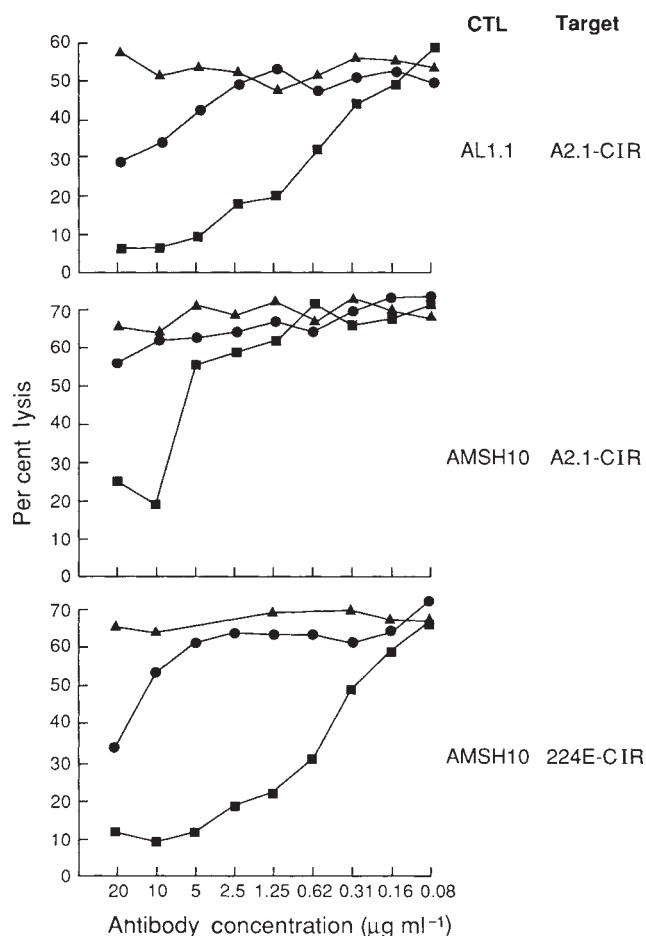
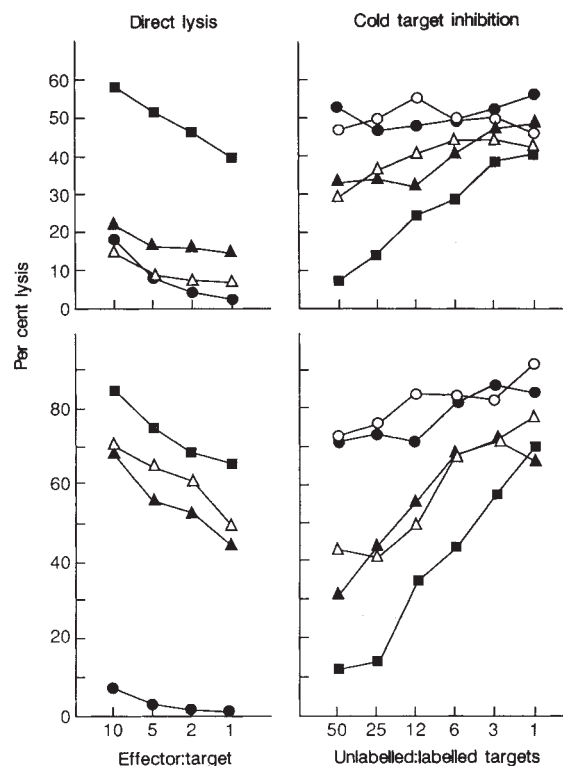


FIG. 6 CD8 and T-cell receptor binding sites on the same class I molecule are required for efficient lysis by a CD8-dependent CTL. *a*, Lysis by clone AL1.1 of A2.1-CIR (■), 245V-CIR (▲), 245V-CIR co-transfected with HLA-B7 (△), and untransfected CIR (●) at varying E:T. In the experiment displayed, no specific binding of the 245V-CIR transfectants to CD8 expressing CHO cells was obtained, whereas 50% of the doubly transfected cells bound. Expression of 245V in the double transfectant was 66% of that in the single transfectant; the total expression of class I molecules on the double transfectant was 122% of that of the single transfectant. *b*, Lysis of the same targets by the bulk culture AJY. *c*, A2.1-CIR lysis by AL1.1 (E:T=5) in the presence of unlabelled competitor target cells. Symbols designate the same cell lines as in (*a*) with the addition of HLA-B27-CIR (○). *d*, AJY lysis of A2.1-CIR (E:T=5) with the same panel of unlabelled competitor cells. METHODS. Unlabelled competitor cells were added at the same time as ^{51}Cr labelled targets to effector CTL. The assay was then continued as described in Fig. 4.



not supplant T-cell recognition of suboptimal amounts of the specific class I molecules^{39,40}.

Concluding remarks

These studies on HLA-A2 and others on H-2D^d (refs 30–32) demonstrate that the immunoglobulin-like domains of the class I MHC molecule are not just an inert structural support for the antigen recognition site. They show that the α_3 domain provides a site of interaction with the α chain of the CD8 glycoprotein that is important for the recognition of antigen by T cells. Moreover, our results are consistent with non-specific adhesion between class I molecules and CD8 contributing to T cell–target interactions and provide support for the co-receptor model in which simultaneous interaction of CD8 and TCR with complexes of antigen and class I molecules is critical for T-cell activation^{14,16,26}. Our finding that lowered class I affinity for CD8 increases susceptibility to anti-CD8 antibody blockade demonstrates that CD8 independence or dependence are not absolute properties of individual T cells. This suggests that CTL differ not in their requirement for CD8 function, but in the ease with which the complexes of TCR, CD8 and class I MHC molecule are either disrupted or prevented from forming by anti-CD8 antibodies.

An exposed loop involving residues 223–229 appears to be the major contact site of the α_3 domain with the CD8 α chain. This must, however, be considered a minimal CD8-binding site. β_2 -m has a similar structure to the α_3 domain²⁷ and may also contribute contacts with CD8; although no data addresses this question as β_2 -m has been invariant in all the class I molecules we studied. Neither have interactions between the CD8 β -chain and class I MHC molecules been investigated. One interesting possibility is that CD8 α interacts with α_3 and CD8 β with β_2 -m. Changing the ratio of CD8 homodimers and heterodimers on the T-cell surface could then change the affinity of CD8 for individual class I molecules and the capacity for CD8-mediated cross-linking of different class I molecules. Equally intriguing is the question as to whether CD4 interacts with class II MHC molecules in a manner homologous to the class I–CD8 interaction. □

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Inflation and the Ω -problem

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OBSERVATIONAL estimates suggest that the cosmological density parameter Ω , defined as the ratio of the density of the Universe ρ_0 and the critical density ρ_{crit} which divides open from closed universes, is ≤ 0.5 . According to the standard Friedmann models, any value of $\Omega \neq 1$ diverges away from one as the Universe evolves. The cosmological Ω -problem is that, on the one hand, making the present value of Ω close to but not equal to one requires an extraordinarily precise adjustment of the initial conditions of the Universe¹, but on the other hand inflationary models, which were devised in part to avoid this fine-tuning, predict that Ω should differ from one only by an exponentially small amount². Conventional inflationary models thus appeal to the existence of 'missing' matter or a non-zero cosmological constant to make up the density deficit. Here it is shown that 'extended inflation'^{3,4}, a recent variation on inflationary cosmology, accommodates a range of initial conditions which lead to $\Omega \leq 0.5$. The parameter range is narrow, perhaps finely tuned, but non-zero.

In inflationary cosmologies of all kinds, the Universe spends a period of time in its early history in a supercooled state, when its density is dominated by a large positive and constant vacuum energy density. The consequent exponential expansion drives Ω towards one, rather than away from it, and requires Ω to be very close to one even now. In addition, inflation blows up a small, causally connected region of the Universe into something much bigger than the observable Universe today; this naturally produces large-scale cosmological homogeneity, and also reduces to an exponentially small number the present density of any magnetic monopoles that, according to many Grand Unified Theories of particle physics, would have been copiously produced in the pre-inflationary phase of the Universe.

In 'old inflation', the supercooled state was marked by the appearance of 'bubbles' of the true vacuum, the broken-symmetry ground state. This model failed because the exponential expansion of the supercooled state prevents the bubbles from merging and completing the phase transition. In 'new inflation'^{5,6}, a single bubble inflates to encompass the entire observable Universe, and the small fluctuations within it may lie behind galaxy formation. It has been argued^{7,8} that in new inflation suitable fine-tuning can permit the present value of Ω to be substantially different from one; I first argue that this is not so.

The time evolution of Ω in a Friedmann-Robertson-Walker Universe is

$$\Omega = 1 + \frac{k}{R^2 H^2} \quad (1)$$

where $R(t)$ is the scale factor, $H \equiv \dot{R}/R$ is the Hubble parameter and $k = +1, -1$ or 0 if the Universe is closed, open or flat. For the case that the present value, Ω_p , is < 0.5 , $k = -1$. The relationship between Ω_b , the value at the beginning of inflation, and Ω_p is

$$1 - \Omega_p = \frac{H_b^2 R_b^2}{H_p^2 R_p^2} (1 - \Omega_b) \\ = \left(\frac{H_b^2 R_b^2}{H_e^2 R_e^2} \right) \left(\frac{H_e^2 R_e^2}{H_p^2 R_p^2} \right) (1 - \Omega_b) \quad (2)$$

where R_e and H_e are evaluated at the end of the inflationary epoch. During inflation, H is nearly constant and $R_e/R_b \equiv e^N$, where N is the number of e-foldings of inflation. After the inflationary transition, $H \propto T^2/M_{\text{pl}}$ and $R \propto 1/T$, where T is the temperature of the radiation and $M_{\text{pl}} = 1.2 \times 10^{19}$ GeV is the

Planck mass. Substituting these expressions into equation (2) and using the fact that $1 - \Omega_b \leq 1$, it is found that the right-hand side is bounded above by $\exp[-2N + 2 \ln(T_e/T_p)]$. Therefore, if Ω_p^{obs} is the present observed value

$$N \leq \ln(T_e/T_p) - \frac{1}{2} \ln(1 - \Omega_p^{\text{obs}}) \quad (3)$$

For $\Omega_p^{\text{obs}} \leq 0.5$, $T_p \sim 3 \text{ K} \sim 10^{-13} \text{ GeV}$ and $T_e \leq 10^{17} \text{ GeV}$, $N \leq 68$.

Further, it is necessary that N be large enough to resolve the horizon, monopole and density fluctuation problems. As demonstrated below, the horizon and monopole problems can be resolved for values of N consistent with $\Omega_p \leq 0.5$ (ref. 7). But the density fluctuations constraint rules out such small values of N in conventional new-inflation models, whereas density fluctuations place no constraint on N in extended-inflation models.

The horizon problem stems from the fact that, according to the standard Big Bang model, the cosmic microwave background radiation last scattered from regions that were never in causal contact. Consequently, the observed uniformity in the microwave-background temperature is inexplicable. The horizon problem is solved if there is sufficient inflation such that regions of last-scattering were in causal contact before inflation. That is, the light travel distance (in co-moving coordinates) before inflation should exceed the distance traversed by the microwave background since decoupling, t_d (ref. 7)

$$\int_{t_d}^{t_p} \frac{dt}{R(t)} < \int_0^{t_b} \frac{dt}{R(t)} \quad (4)$$

The integral on the left side is $\sim 2/(R_p H_p) = 2\sqrt{(1 - \Omega_p)}$ (see equation (1)). If the Universe is radiation-dominated in the pre-inflation epoch $R(t) = [(2H_b R_b^2 \Omega_b^2)t - kt^2]^{1/2}$ and equation (4) reduces to

$$2\sqrt{(1 - \Omega_p)} < \cosh^{-1}(1/\Omega_b^{1/2}) \quad (5)$$

Equation (5) can be used to express Ω_b in terms of Ω_p . By substituting the relation into equation (2), the horizon constraint is found to be satisfied if

$$N \geq \ln(T_e/T_p) - \frac{1}{2} \ln(1 - \Omega_p^{\text{obs}}) - \gamma \quad (6)$$

where $\gamma \equiv -\frac{1}{2} \ln(1 - \Omega_b)$ takes values $0.045 < \gamma < 0.12$ for $0.1 < \Omega_p^{\text{obs}} < 0.5$ according to equation (5). Hence, a narrow range of N exists such that $\Omega_p \leq 0.5$ (see equation (3)), and yet the horizon problem is solved (equation (6)).

The monopole problem is resolved if there is sufficient inflation to reduce the monopole mass density below the baryon mass density. Without inflation, particle-physics models predict a monopole mass density of the order of $M_m H_m^3$, where M_m is the monopole mass and H_m is evaluated at the time of monopole production². This density is sufficient to dominate the energy density of the Universe, altering the expansion in a way that destroys the successful predictions of the Big Bang model. The baryon mass density is $\sim (1 \text{ GeV}) H_{\text{ba}}^3$, where H_{ba} is evaluated at the time of baryon-asymmetry production. Assuming $H_m \geq H_b > H_{\text{ba}}$, inflation can dilute the ratio by a factor of e^{-3N} ; the monopole problem is resolved if

$$N \geq \frac{1}{3} \ln \left[\frac{M_m}{1 \text{ GeV}} \right] + \ln \left[\frac{H_m}{H_{\text{ba}}} \right] \quad (7)$$

the right-hand side is typically between 15 and 30. Hence, the monopole problem results in a less stringent limit on N than the horizon problem.

The critical constraint on N is that the amplitude of density fluctuations produced during inflation, $\delta\rho/\rho$, must be less than 10^{-4} in order to match the observed isotropy of the microwave background. The predicted amplitude depends on the details of the inflationary phase transition. The original, 'old inflation' model assumed a strongly first-order phase transition. If the Universe supercools into a metastable or 'false vacuum' phase (such as that associated with some phase transition predicted

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by particle physics), it can be prevented from reaching a lower-energy phase if there is an energy barrier. The energy density of the false phase then acts as an effective cosmological constant, leading to the desired inflationary expansion. The flaw is that the inflationary expansion cannot be stopped. Spontaneous quantum fluctuations will cause some regions to tunnel through the barrier, producing bubbles of true phase that expand and convert the Universe from false to true vacuum. But the remaining false vacuum regions expand too rapidly compared to bubble nucleation and expansion, and so the physical volume of false phase increases forever.

'New inflation' finesses the 'graceful exit' problem by assuming a special phase transition in which the barrier disappears as the Universe supercools to low temperature. At this point, small fluctuations can push the Universe away from the false vacuum, and it can evolve smoothly to the true phase. The inflationary expansion stops once the Universe evolves to the true phase. Inflation is governed by the evolution of an 'inflaton' field, ψ , the average value of which is zero in the false vacuum phase and non-zero in the true vacuum phase. Its evolution from the false to the true vacuum is determined by its effective potential, $V(\psi)$, which depends on the coupling between ψ and other particle fields. New inflation requires that $V(\psi)$ be very flat near $\psi = 0$ and monotonically decrease to a minimum at the true-vacuum value of ψ . The equation of motion for ψ is (ref. 6)

$$\ddot{\psi} + 3H\dot{\psi} + V'(\psi) = 0 \quad (8)$$

Inflation occurs as ψ 'slow-rolls' down the flat portion of $V(\psi)$ near $\psi = 0$ where the friction term (the second term of equation (8)) dominates. The slow-roll range corresponds to $\dot{\psi}/3H\dot{\psi} < 1$. In this limit, $V''(\psi) < 9H^2$ and $\dot{\psi} = -V'(\psi)/3H$. For the purposes of discussion, I consider a new-inflation model with $V = V_0 - \lambda\psi^n$ in the slow-roll region of the potential and an initial value of $\psi = 0$. Slow-roll is initiated when small de Sitter fluctuations push ψ to $\psi_b \approx H \approx V_0^{1/2}/M_{\text{Pl}}$ (ref. 6).

The number of e-foldings of inflation is⁹

$$N \equiv \int_{t_b}^{t_e} H dt = \int_{\psi_b}^{\psi_e} \frac{3H^2 d\psi}{V'} \approx \frac{3H^2}{V''(\psi_b)} \quad (9)$$

where ψ_e is the value at the end of inflation. To constrain N , I note that $\delta\rho/\rho \approx H^2/\dot{\psi}_h$ must be less than 10^{-4} , where $\dot{\psi}_h$ is evaluated when the present horizon was stretched beyond H^{-1} during the inflationary epoch, roughly 60 e-foldings before the end of inflation¹⁰⁻¹³. That is, if ψ began at ψ_h rather than at ψ_b , then (adapting equation (9))

$$60 \approx \frac{3H^2}{\lambda n(n-1)\psi_h^{n-2}} \quad (10)$$

Note that ψ_h also lies in the slow-roll region of equation (8), and so $\dot{\psi}_h = V'(\psi_h)/3H = \lambda n\psi_h^{n-1}/3H$. The constraint on $\delta\rho/\rho$ can then be recast as

$$\frac{\delta\rho}{\rho} \approx \frac{H^2}{\dot{\psi}_h} = \left(\frac{3H^2}{\lambda n(n-1)\psi_h^{n-2}} \right) \left(\frac{(n-1)H}{\psi_h} \right) < 10^{-4} \quad (11)$$

The first factor is ~ 60 according to equation (10), so $\psi_h > 6 \times 10^5 (n-1)H$. The ratio of equation (9) to equation (10), using $\psi_b \approx H$, yields the key result

$$N/60 = (\psi_h/\psi_b)^{n-2} = [6 \times 10^5 (n-1)]^{n-2} \quad (12)$$

For $n > 2$, the constraint on the fluctuation amplitude requires $N \gg 100$, which makes it impossible to satisfy equation (3). (The case where $n \leq 2$ needs more careful analysis, but yields the same qualitative conclusion. See Table I in ref. 9.) Thus, conventional inflationary models that satisfy the density fluctuation constraint are inconsistent with $\Omega \leq 0.5$ (ref. 7). (In principle, $\Omega \leq 0.5$ is possible in 'chaotic inflation'¹⁴. This requires, however, that the observable Universe evolved from a region of space in which the ψ field and all its time-derivatives chaotically fluctuated to specially chosen values.)

Density fluctuations place no constraint on N in the recently proposed 'extended inflation' models.^{3,4} In extended inflation, a special phase transition is not needed. That is, $V(\psi)$ can have a non-negligible barrier separating the true and false vacuum phases. The flaws of old inflation are avoided if the effective gravitational constant, G , varies with time during inflation. Time-varying G slows the inflationary expansion from exponential to power-law dependence in time. The slower, power-law expansion permits a first-order inflationary transition to be completed by ordinary bubble nucleation.

For G to vary with time during the inflationary epoch, it is sufficient that there be some field ϕ (different from ψ) which is non-minimally coupled to gravity. The action would have the form^{3,4}

$$A(\phi, \psi) \equiv \int d^4x (-g)^{1/2} \{ -\xi \phi^2 \mathbf{R} + \frac{1}{2} \partial_\mu \phi \partial^\mu \phi - W(\phi) - M_{\text{Pl}}^2 \mathbf{R} + 16\pi L_{\text{matter}}(\psi) \} \quad (13)$$

where $\mathbf{R}$ is the curvature, ξ is the non-minimal coupling constant and $W(\phi)$ is the effective potential for the ϕ field. (A major improved version, 'hyperextended inflation', has been proposed which allows more general non-minimal couplings and works for a much wider range of parameters¹⁵.) $L_{\text{matter}}(\psi)$ contains the contributions of all other fields, including the ψ field that drives the inflationary phase transition. The Universe is assumed to supercool into the false vacuum of $V(\psi)$ with energy density $\rho_F \equiv M_F^4$. Inflation is completed by the spontaneous nucleation of bubbles of true vacuum which coalesce and reheat the Universe to a temperature $T_e \approx M_F$.

The requisite action, equation (13), is found in a wide variety of theories. This includes non-Einstein theories of gravity in which G varies for all cosmological time. For example, if $V(\phi)$ and M_{Pl} are set to zero, the theory can be recast as a Brans-Dicke theory with Brans-Dicke parameter $b = 1/8\xi$ (ref. 16). Similar forms are found in Kaluza-Klein models¹⁷ or theories that include dilaton fields (superstrings¹⁸, for example). It should be emphasized, however, that extended inflation does not require such a radical departure from conventional gravitation theory. If M_{Pl} and $V(\phi)$ are non-zero and $V(\phi)$ has a minimum at some non-zero value of ϕ , the action describes ordinary Einstein gravity once ϕ reaches its minimum. But during the inflationary epoch ϕ is driven away from its minimum and varies in a manner similar to a Brans-Dicke field. The coupling of the ϕ -field to the curvature results in a time-varying contribution to the effective value of G . The time variation may be sufficient to complete the phase transition. Once the phase transition is completed, ϕ sinks back to its minimum, and standard Einstein gravity is recovered. As non-minimally coupled fields are expected in nearly all particle field theories, introducing a time-varying G in this manner seems quite natural and consistent with all experimental bounds.

During the inflationary epoch when $L_{\text{matter}} = \rho_F$, ϕ evolves from some initial value m_{Pl} as $\phi(t) = (m_{\text{Pl}}/\sqrt{\xi})(1 + H_b t/\alpha)$, where $H_b \equiv (8\pi/3)^{1/2} M_F^2/m_{\text{Pl}}$ is the Hubble parameter at the beginning of inflation, $t_b = 0$ (ref. 3). As justified below, $\xi \ll 1$ can be assumed; in this limit, $\alpha \approx 1/8\xi$. See ref. 4 for exact expressions. After inflation ends at $t = t_e$, ϕ does not evolve significantly (if $\xi \ll 1$) in the subsequent radiation- or matter-dominated epochs. Hence, I can take $\phi(t_e) = M_{\text{Pl}}/\sqrt{\xi}$. Given that $R(t) \propto \phi^{1/8\xi}$ during inflation

$$N = \ln \left[\frac{R_e}{R_b} \right] = \ln \left[\left(\frac{\phi(t_e)}{\phi(t_b)} \right)^{1/8\xi} \right] = \frac{1}{8\xi} \ln \left[\frac{M_{\text{Pl}}}{m_{\text{Pl}}} \right] \quad (14)$$

The critical result is that N depends on ξ and m_{Pl} , but not on M_F .

There are two different contributions to the density fluctuation amplitude in extended inflation⁴. First, fluctuations due to variations in ϕ during inflation, $\delta\rho/\rho = H^2/\dot{\phi}_h$, result in the constraint (see equation (14) of ref. 4)

$$\frac{\delta\rho}{\rho} \approx \left(\frac{8\pi}{3}\right)^{1/2} \left(\frac{M_F}{M_{\text{Pl}}}\right)^2 \left(\frac{M_F}{T_p}\right)^{16\xi} \leq 10^{-4} \quad (15)$$

Second, there are fluctuations due to bubble walls. When inflation ends, the energy contained in the bubble walls thermalizes as bubbles collide and begins to flow back into the bubble interior. For bubbles larger than the horizon volume at decoupling, there has not been adequate time since inflation for the thermal energy to flow back into the interior. To avoid inhomogeneities that conflict with observed isotropy of the microwave background^{4,19}, the fractional volume of such large bubbles must be less than 10^{-4} . According to ref. 4, this can be satisfied for $10^2 \text{ GeV} < M_p < 10^{19} \text{ GeV}$ and $0.08 > \xi > 0.005$. As promised, the density fluctuation constraint and N are decoupled in extended inflation: for a given ξ between 0.08 and 0.005, it is possible to choose M_F to satisfy the density fluctuation constraint, and m_{Pl} can be chosen independently to adjust N . In hyperextended inflation models¹⁵, the same result holds for a much wider range of ξ and M_F .

Finally, how must the parameters of extended inflation (ξ , M_F , m_{Pl}) be chosen so that $\Omega_p \leq 0.5$? I have found that N must be $\sim \ln(T_c/T_p)$ to obtain $\Omega_p \leq 0.5$ and solve the horizon and monopole problems. From equation (9), $m_{\text{Pl}} = M_{\text{Pl}}(T_p/M_F)^{8\xi}$. The fluctuations constraint, equation (15), requires $M_F \leq 10^{-3} M_{\text{Pl}} T_p^{8\xi}$. Together, these conditions imply $m_{\text{Pl}} \geq 10^3 M_F$; for example, $M_F \approx 10^{14} \text{ GeV}$, $\xi = 0.01$ and $m_{\text{Pl}} \approx 10^{17} \text{ GeV}$ – physically reasonable magnitudes for these parameters.

To be fair, I have only established that extended inflation is possible with $\Omega_p \leq 0.5$, and not that $\Omega_p \leq 0.5$ is likely. It is encouraging that magnitudes of the parameters required to obtain $\Omega \leq 0.5$ are physically reasonable, but the absolute range is rather narrow. The replacement of exponential inflation with power-law inflation helps by making the range more plausible because N increases more slowly as a function of parameters, but the range is still a small fraction of the allowed parameter space. Another objection might be that, if $\Omega_p \neq 1$, spatial variations in the pre-inflation value of Ω_p should be reflected in spatial variations in Ω_p today. Unless the pre-inflation variations are small, perhaps the consequent variations in Ω_p could unacceptably distort the microwave background. It is difficult to judge whether small, pre-inflation fluctuations are to be expected, especially as these fluctuations would be on scales over which there was causal contact during the pre-inflation epoch. Ultimately, computer simulations of the evolution of the variations before and during the inflationary epoch are required to address the issue. Whereas these issues were moot previously because conventional inflation models required so many e-foldings of inflation ($N \gg 68$), extended inflation eliminates this problem and, hence, invites further critical evaluation. $\square$

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Chaotic motion in a primordial comet disk beyond Neptune and comet influx to the Solar System

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CHAOTIC dynamics, characterized by extremely sensitive dependence on initial conditions with exponential divergence of neighbouring trajectories and subsequent loss of predictability¹, have recently been demonstrated to be quite common in the Solar System. The long-standing problems of the delivery of meteorites to Earth-crossing orbits² and the existence of the Kirkwood gaps³ have essentially been solved by the discovery of the chaotic zones with divergence timescales of a few million years in the asteroid zone near orbital resonances of Jupiter^{3–7}. It has also been demonstrated⁸ that the orbit of Pluto is chaotic with divergence timescales of ~ 20 Myr. Numerical mapping techniques, which underestimate chaotic behaviour, have determined that orbits lying between the outer planets can exhibit chaotic motions⁹, and recent numerical studies¹⁰ have indicated that even the inner planets exhibit chaotic motion with divergence timescales of ~ 5 Myr. Previous calculations¹¹ using a heliocentric frame have shown that a scattered disk of cometsimals beyond Neptune develops chaotic motions with divergence timescales less than 1 Myr. Here we describe improved numerical simulations using a barycentric system and thus including the additional frequencies associated with motion of the Sun. Because the onset of chaotic motion is characterized by the appearance of broad-band noise, the inclusion of these additional perturbations should increase the likelihood of stochastic behaviour and thus the chaotic zone should be larger. Our results indeed indicate that comets in a low-eccentricity primordial disk beyond Neptune can exhibit chaotic motions which lead to interesting consequences for the dynamics of comets.

We have undertaken an extensive numerical program of direct integration of the equations of motion for disk comets to assess

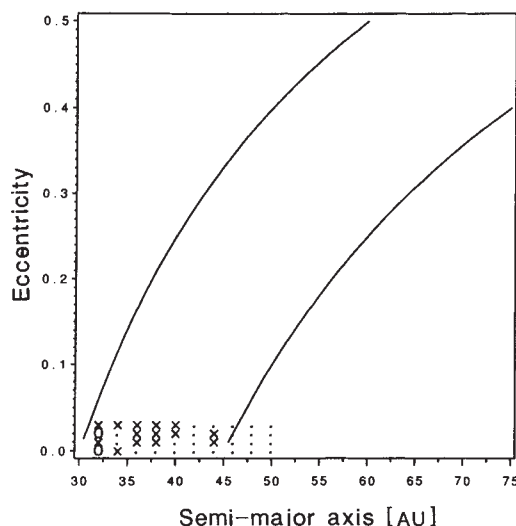


FIG. 1 Initial semi-major axes and eccentricities of a thin primordial disk of cometsimals beyond Neptune and the results of 10-Myr integrations of their motion. Symbols denote the character of the motion: dots denote regular motion, $\times$ denotes chaotic, and $\circ$ denotes chaotic and Neptune-crossing. Solid lines enclose the region for which the perihelion lies in the range 30–45 AU.

the extent of the chaotic zone beyond Neptune. The experimental procedure involved direct integration of the three-dimensional equations of motion of comets in an inertial barycentric coordinate system subject to the perturbations of all four major planets. These planets were constrained to move in fixed orbits with their present orbital elements. No attempt was made to incorporate the dynamical effect of variations of the planetary elements resulting from mutual interactions. Inclusion of these motions would introduce additional frequencies and thus tend to increase the size of the chaotic region. The four major planets were assigned masses and orbital elements (semi-major axis a , eccentricity e , inclination i , longitude of perihelion $\tilde{\omega}$, longitude of ascending node Ω) taken from Allen¹², and orbital elements for comets were then chosen for various values of the semi-major axis and eccentricity with $\tilde{\omega} = \pi$ and $i = \Omega = 0$. The equations of motion for a given comet in cartesian coordinates are given by

$$\ddot{\mathbf{x}} = -\frac{GM_{\odot}(\mathbf{x}-\mathbf{x}_{\odot})}{r^3} - \sum_i \frac{GM_i(\mathbf{x}-\mathbf{x}_i)}{d_i^3} \quad (1)$$

where $\mathbf{x} = (x, y, z)$ and the summation index i runs over the four major planets, $r = \|\mathbf{x} - \mathbf{x}_{\odot}\|$ is the distance of the comet from the Sun, $d_i = \|\mathbf{x} - \mathbf{x}_i\|$ is the distance between the comet and the i th planet, and $M_{\odot}$ and M_i are the masses of the Sun and the planet respectively. These equations were integrated for 10 Myr in double-precision arithmetic using a Runge-Kutta scheme with variable step-size (RK78) and a tolerance set at 10^{-12} . Relative errors in the energy for unperturbed motion amounted to values of the order of 10^{-8} at the end of the calculations.

The stability of the resulting motion was assessed by calculating the value of the principal Lyapunov exponent, γ , which measures the rate at which neighbouring trajectories diverge, using the rescaling technique^{13,3}. For regular or quasi-periodic motion, $\gamma \rightarrow 0$ as $t \rightarrow \infty$. For chaotic motion, however, γ tends to a positive constant as $t \rightarrow \infty$. The time dependence of γ was assessed in a manner described elsewhere¹¹.

A primordial disk of comets¹⁴⁻¹⁶ would be characterized initially by small random motions. The mean eccentricity expected for an accreting planetesimal swarm beyond Neptune containing cometesimals ~ 100 km in diameter is several times 10^{-2} (ref. 17). Accordingly, for the calculations we chose initial semi-major axes and eccentricities of forty primordial comets uniformly between 32–50 AU and 0–0.03, respectively, as shown in Fig. 1.

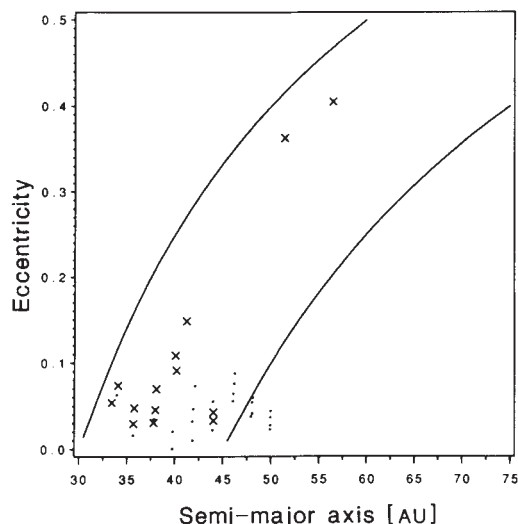


FIG. 2 Final distribution of semi-major axes and eccentricities after 10 Myr for the comets of Fig. 1. Symbols as in Fig. 1. Note that chaotic trajectories tend to diffuse to larger values of semi-major axis and eccentricity, forming a scattered disk.

The character of the motion after 10 Myr of integration is also shown in Fig. 1. Trajectories exhibiting regular motion are denoted as dots, and chaotic trajectories are depicted as crosses. Trajectories that become Neptune-crossing are depicted as circles. The solid lines bound a region satisfying the constraint that the perihelia lie in the range 30–45 AU, which constitutes a low-eccentricity extension of the chaotic zone found previously in a heliocentric coordinate system¹¹. As can be seen, a significant number of trajectories within a primordial disk can exhibit chaotic dynamics. Because of these chaotic motions, objects in the comet disk that are originally on orbits that would not intersect Neptune's orbit can evolve to orbits that do. In subsequent encounters with Neptune and Uranus these comets can, according to the standard model¹⁸⁻²⁰, be scattered to the inner Solar System and outwards to larger semi-major axes or ejected. Thus, the primordial disk can serve as a source for some of the comets scattered to the Oort cloud as well as objects participating in planet accumulation.

Another effect of chaotic dynamics on orbits in this region of the Solar System is that some of the chaotic orbits tend to diffuse to larger values of semi-major axis and eccentricity, as can be seen in Fig. 2 which shows a and e at the end of the calculation for the initial conditions plotted in Fig. 1. A small fraction, about 0.1, of the chaotic trajectories are observed to random-walk through the chaotic zone along paths that maintain roughly constant perihelion. Based on the limited statistics available, orbits sent to large semi-major axes and eccentricities also tend to be sent to larger perihelia. Thus, a significant fraction of the chaotic trajectories can be temporarily removed from the strongest planetary interactions and may ultimately be driven to longer-lived orbits with large perihelia. Hence, a sizeable portion of the primordial disk can be scattered to more distant regions and effectively stored.

To estimate the subsequent behaviour of objects scattered to larger semi-major axis and eccentricity, 10-Myr integrations were performed for over 150 comets in a much larger region of a, e space. The results are shown in Fig. 3, which is similar to Fig. 1 in that initial positions are plotted. The region between the lines of constant perihelion of 30 and 45 AU is seen to adequately characterize the chaotic zone. Cometary orbits random-walking along lines of roughly constant perihelion may, therefore, eventually be scattered from a primordial disk to a scattered disk roughly congruent with the chaotic zone of Fig. 3. Once in place, such a scattered disk could provide a

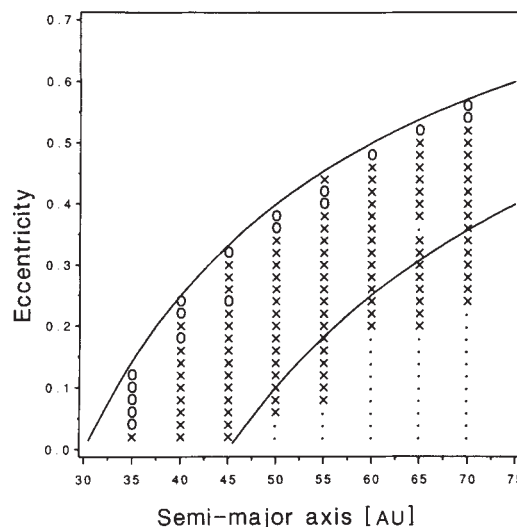


FIG. 3 Results of 10-Myr integrations for a scattered disk of comets, showing the extent of the chaotic zone. Symbols as in Fig. 1. Comets sent to this scattered disk can thus be eventually returned to the inner Solar System.

reservoir of comets for later injection to Neptune-crossing orbits.

Assuming equilibrium diffusion of trajectories sent to high a and e , one can estimate the timescale over which the population sent to storage becomes substantially depleted. Of those trajectories that become Neptune-crossing, roughly one-third (those closest to the upper line in Fig. 3) were scattered inwards in less than 0.5 Myr. The rest, originating with larger perihelia, became Neptune-crossing, given the limited statistics, at a rate not inconsistent with a constant flux. Not counting those injected immediately, $\sim 10\%$ of the remaining chaotic trajectories— $\sim 7\%$ of the total population in the scattered disk—become Neptune-crossing in 10 Myr. If we assume that, on average, a fraction ~ 0.07 become Neptune-crossing every 10 Myr, we obtain a very rough estimate for the lifetime of this flux, $\tau \approx 140$ Myr.

One might realistically expect, however, that primordial disk comets are scattered to larger semi-major axes with a range of perihelia and thus, based on the present limited results, a range of Neptune-crossing timescales. Hence, the flux of comets to the inner Solar System could conceivably extend for several hundred Myr and may account for some of the projectiles responsible for the late heavy bombardment of the terrestrial planets²¹. If the population in the scattered disk is at least comparable to that in the Oort cloud, $\sim 10^{12}$ – 10^{13} , and if only $\sim 1\%$ of this disk reaches the inner Solar System over several hundred Myr, the result is a mean flux of $\sim 10^2$ – 10^3 comets per year.

Chaotic orbital diffusion within the scattered disk can, in principle, store comets in regions with Neptune-crossing timescales comparable to the age of the Solar System. Their ultimate return, together with direct injection from the primordial disk from $a > 50$ AU may, therefore, account for the present flux of short-period comets¹¹. $\square$

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Young asteroid melting event indicated by Rb–Sr dating of the Point of Rocks meteorite

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THE record of collisions on asteroids, as preserved in meteorite breccias, provides important constraints on impact cratering events in the inner Solar System^{1,2}. Many young gas-retention ages (with a peak at ~ 500 Myr) have been reported for meteorite breccias^{3,4}, but except for a few cases⁵, they have not been substantiated by the much less susceptible clocks based on refractory parent-daughter nuclides such as ^{87}Rb – ^{87}Sr . Furthermore, the nature and intensity of the thermal events responsible for young meteorite ages have not been well evaluated. Here we report an age of 460 ± 11 (2σ) Myr, well defined by a Rb–Sr internal isochron for the completely impact-melted L-chondrite, Point of Rocks. This age provides the first evidence for a young intense collision leading to melting on an asteroid.

Points of Rocks is an L-chondrite that has been heavily shocked and has undergone subsequent recrystallization^{6,7}. The meteorite includes relatively clast-free, impact melt regions (5–10 cm across) which contain 10–30 vol% of shock melt. As a part of consortium study on melt-rock breccias led by Professor K. Keil (University of New Mexico), we have undertaken Rb–Sr isotope and trace element (including rare-earth elements (REEs)) analyses for the Point of Rocks meteorite. A piece (2.5 g) of the meteorite specimen (sample number C78.11a) investigated in this work was taken from a total-melt region (K. Keil, personal communication). The specimen examined in this work has microporphyrict texture consisting of poikilitic clinopyroxene enclosing olivine grains which might have crystallized from a melt. It contains numerous rounded metal-troilite particles ranging in size from several to 10 μm . The general

textural features that we observed are consistent with those reported by Scott *et al.*⁷.

Three whole-rocks and seven mineral separates have been analysed for Rb–Sr isotopic compositions and concentrations. Concentrations of Mg, Fe, Ca, K and REEs also were determined by direct-loading or conventional mass spectrometric isotope dilution analyses. The analytical techniques used in this work have been published elsewhere^{5,8}. In Table 1, three whole-rock samples are shown to have similar Mg and Fe concentrations, which may reflect the uniform compositions of olivine and pyroxene formed by rapid crystallization⁷, but show a relatively large variation (up to a factor of 2.6) for K. The abundances of Mg, Ca, Rb and Sr in the whole-rocks are mostly within the range for L-group chondrites^{9,10} but the Fe abundances (17.3–19.8%) are systematically lower than typical L-chondrites (20.2–23.6%)¹⁰, suggesting that the melted fraction of Point of Rocks had lost some of its metal-troilite during the impact melting. No particular influence is noted on the REEs distribution pattern of the meteorite, however, except for a minor positive Eu anomaly.

To avoid potential selective leaching of Rb relative to Sr, heavy liquids were not used for mineral separation. Instead, magnetic separation with a Franz Isodynamic Separator was used. The existence of fine-grained metal particles that were injected into olivine and pyroxene grains and also into glassy materials made a clean mineral separation extremely difficult. The Rb–Sr concentration analyses, however, reveal enough variation of Rb concentration (a factor of 11) so that in spite of relatively constant Sr a large spread (ranging from 0.16 to 1.8) of $^{87}\text{Rb}/^{86}\text{Sr}$ ratio was obtained (Table 1). The K is positively correlated with Rb for most samples, including one whole-rock (B). These samples have Rb/K ratios similar to those of typical L-chondrites. Two whole-rocks (A and C) indicate unusually low Rb/K ratios, suggesting that K-rich (relative to Rb) components exist heterogeneously in the meteorite but were not sampled in the mineral separates we analysed.

A ^{87}Rb – ^{87}Sr evolution diagram is shown in Fig. 1. Most data points form an array crossing the 4,550-Myr evolution line corresponding to the Allende initial $^{87}\text{Rb}/^{86}\text{Sr}$ ratio¹¹. The 10 data points define a best-fit straight line, which yields an age of 461 ± 11 (2σ) Myr and an initial $^{87}\text{Rb}/^{86}\text{Sr}$ ratio of $0.74965 \pm$

TABLE 1 Results of isotope and concentration analyses for the Point of Rocks meteorite

Sample	Weight (mg)	Mg (wt%)	Fe (wt%)	Ca (wt%)	K (p.p.m.)	Rb (p.p.m.)	Sr (p.p.m.)	$^{87}\text{Rb}/^{86}\text{Sr}^*$	$^{87}\text{Sr}/^{86}\text{Sr}^\dagger$
Whole rock									
WR-A	53.02	14.6	19.8	1.21	1,176	2.10	10.68	0.571	0.752801 ± 63
WR-B	52.12	14.4	19.0	1.09	599	2.04	8.18	0.723	0.754381 ± 52
WR-C	52.16	14.9	17.3	1.28	1,536	1.20	10.47	0.333	0.751917 ± 118
Mineral concentrates ($200 > \phi > 300$ mesh)									
C1 (more magnetic)	14.50	16.6	17.0	1.20	1,243	5.17	8.32	1.806	0.761818 ± 36
C2	35.25	16.1	15.0	1.30	797	2.52	12.39	0.592	0.753569 ± 46
C3	32.06	16.0	14.2	1.44	499	0.944	12.51	0.219	0.751122 ± 52
C4 (less magnetic)	19.86	16.9	13.2	1.86	284	0.466	8.42	0.161	0.750847 ± 31
Mineral concentrates ($\phi < 300$ mesh)									
F1 (more magnetic)	16.96	15.9	19.2	0.982	884	3.43	8.46	1.46	0.756839 ± 39
F2	46.05	17.2	18.3	1.08	606	1.82	10.59	0.500	0.752993 ± 48
Fe (less magnetic)	24.98	17.0	14.8	2.06	393	0.869	9.18	0.275	0.751440 ± 38
NBS 987 Sr standard (mean of 11 separate runs of 40–200 ng Sr)									0.710213 ± 22

* Errors are estimated to be $<0.6\%$.

† Ratios are normalized to $^{86}\text{Sr}/^{88}\text{Sr}=0.1194$. Errors correspond to the last digits and 95% confidence level.

The Rb–Sr isotope analyses were done at Kyoto Sangyo University using a mass spectrometer model MAT-261 and concentration analyses of Mg, Fe, Ca and K were carried out using a mass spectrometer model JMS-05RB at Kobe University.

0.00012 (2σ). In spite of the large spread of $^{87}\text{Rb}/^{86}\text{Sr}$, the calculated error in the age is somewhat large, because of the scatter of three data points (two magnetic fractions [F1 and C1] and one whole-rock [A]), as shown in the inset to Fig. 1. We had no analytical problems specific to these samples, but we must consider the potential importance of terrestrial weathering on trace-element behaviour after the meteorite fell. Nevertheless, as all these samples appeared fresh when examined under the binocular microscope, the observed scatter of these three samples is probably intrinsic. Thus, it is likely that the Rb–Sr system of the meteorite was mostly but not totally reset by the thermal event, and that minor amounts of unmelted material such as 'chondritic' components⁷ existed in these samples. Also,

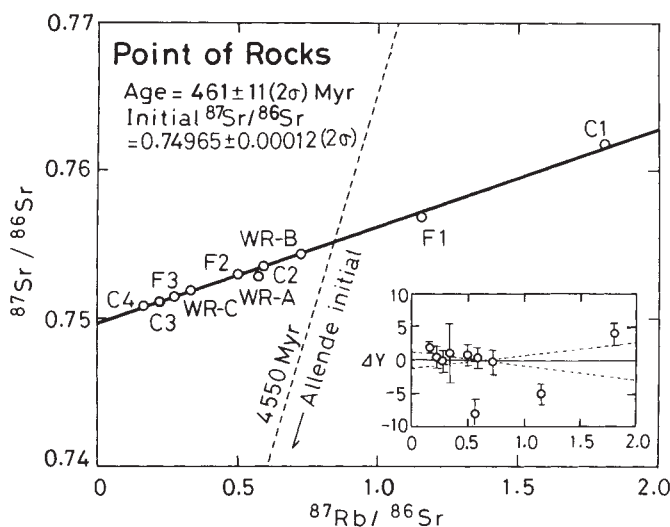


FIG. 1 ^{87}Rb – ^{87}Sr evolution diagram for impact-melts of the Point of Rocks meteorite. The 10 data points of 3 whole rocks (WR-A, -B, -C) and 7 mineral separates ($\phi = 200$ – 300 mesh, C1 [more magnetic], C2, C3, C4 [less magnetic]; $\phi < 300$ mesh, F1 [more magnetic], F2, F3 [less magnetic]) define an isochron, which yields an age of 461 ± 11 (2σ) Myr (using the decay constant of $1.42 \times 10^{-11} \text{ yr}^{-1}$ for ^{87}Rb) and an initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.74965 ± 0.00012 (2σ). The age is interpreted to represent an impact-melting event that occurred on an L-chondritic asteroid. The ΔY in the insert is calculated from the following equation:

$$\Delta Y = \left(\frac{(^{87}\text{Sr}/^{86}\text{Sr})_{\text{measured}}}{(^{87}\text{Sr}/^{86}\text{Sr})_{\text{regressed}}} - 1 \right) \times 10^4$$

data points of the three whole-rocks plot on the left side of the 4,550-Myr line, indicating that a severe Rb/Sr fractionation occurred at the 460-Myr event. Using a two-stage evolution model similar to that of Nakamura and Okano⁵, the Rb loss is estimated to be 15–60% for these three whole-rocks. This calculation and heterogeneous distribution of Rb/K in whole-rocks, as mentioned above, suggest that fractional volatilization of Rb accompanied by local migration of both Rb and Sr occurred during impact melting on the Point of Rocks meteorite parent body.

Many K–Ar ages accumulated so far indicate that collisional events occurred continuously throughout the history of meteorite parent bodies, particularly the L-chondrite parent body. It is interesting to note that most shock ages of chondrites are 300–1,400 Myr^{4,5,12,13}, and clusters in ages around 300–700 Myr^{4,12} (with a peak at ~ 500 Myr) seem to exist. Most of these ages have been considered to represent impact-degassing events. It is thus important that the 460-Myr age obtained from Point of Rocks provides new evidence for the youngest melting episode reported for chondrite breccias. The age is younger than the 1,200-Myr Rb–Sr age reported by us for the similarly shock-melted LL-chondrite, Yamato-790964⁵. It is suggested that young collisional events occurring on an L-chondritic asteroid might have produced melt-ejecta sheets in impact craters⁷ and that the Point of Rocks meteorite sampled such a part of the asteroid during fragmentation processes¹. The age of 460 Myr may thus indicate that melt-producing geological activities in a minor planet, which were induced by intense collisions, may have lasted until 4,100 Myr after the formation of the Solar System. □

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Predictability of anomalous river discharge in Guyana

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ESSENTIAL progress in climate prediction for the tropics has come from using empirically based methods, which combine extensive investigations of general circulation diagnostics with statistical analyses¹⁻³. An immediate challenge for tropical climate prediction is presented by those areas where interannual rainfall variability is chiefly controlled by the anomalous behaviour of well defined quasi-permanent components of the large-scale circulation. Here I present a method which, applied to the Essequibo River in Guyana, predicts 74% of the interannual variance of water discharge in the January–February season of low stand, using as sole predictor the preceding September–October's sea surface temperature anomaly in the equatorial Pacific. The scheme allows the prediction of hydrological and, implicitly, climate conditions, and the results should be helpful in planning water resources and land use.

Northern South America presents a suitable testing region for climate prediction, because it receives much of its rainfall from the convective activity in the intertropical convergence zone (ITCZ), and its interannual variability shows a pronounced association with the southern oscillation^{4,5}. I therefore studied the general circulation mechanisms of extreme climate events in the region, using a series of water discharges from eight catchments as a measure of the hydrometeorological conditions, and went on to develop seasonal prediction methods from these investigations. This report considers the Essequibo catchment in Guyana (Fig. 1) in particular.

Throughout the year, trade winds sweep over the Caribbean Sea into northern South America and across the central American land bridge into the eastern Pacific to meet the cross-equatorial flow from the Southern Hemisphere in the ITCZ, in which convective activity is concentrated⁶. The latitudinal migration and variation of intensity of the ITCZ in the course of the average annual cycle are depicted in Fig. 2a for the longitude of Essequibo. The maximum convection belt passes over the Essequibo catchment on both its northward and southward sweeps, but only the northward passage of this convection maximum in May–June is associated with a rainfall peak. Convection is less intense during the southward sweep in August–September and this is also rather faster. Water discharge reaches its maximum in July (Fig. 2b), lagging somewhat behind the maximal rainfall (Fig. 2a). Thus, the seasonal latitude migration of the ITCZ seems to provide an important control for the annual cycle of rainfall and river discharge.

River runoff gives an integral measure of the hydrometeorological conditions in a catchment as a whole. This is useful, because existing rain-gauge networks are difficult to maintain and do not resolve well the large spatial and temporal variability of tropic rainfall. Over a long time span, runoff is the excess of rainfall over evaporation for the catchment. Typically, rainfall

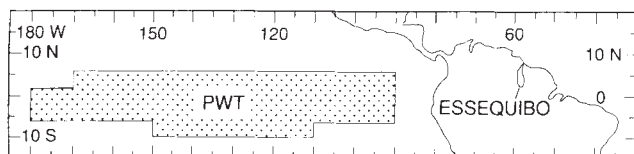


FIG. 1 Orientation map showing location of Essequibo river catchment and domain (2°–6°N, 90°–170°W; 2°N–6°S, 90°–180°W; 6°–10°S, 110°–150°W) of the equatorial Pacific sea surface temperature index, PWT.

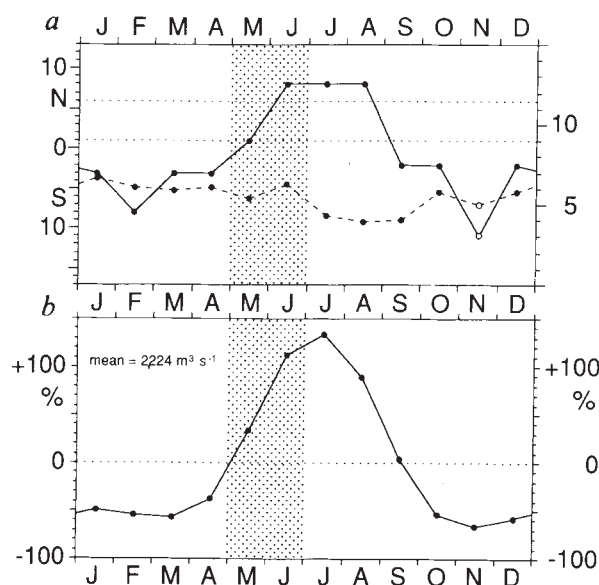


FIG. 2 Annual march of climate in the Essequibo river catchment. Horizontal broken lines indicate latitudinal extent of catchment, and dot raster in vertical column⁸ marks the months of the rainy season peak. *a*, Annual cycles of the maximum of highly reflective clouds (period 1971–87; ref. 9). Solid line and left-hand scale denote the latitude of the maximum (°N and °S), and broken line and right-hand scale, the amount (number of days per month). *b*, Annual cycle of river discharge. Solid lines join monthly values expressed as percentage departures from annual mean of $2,224 \text{ m}^3 \text{ s}^{-1}$ (period 1950–83), indicated by a horizontal broken line.

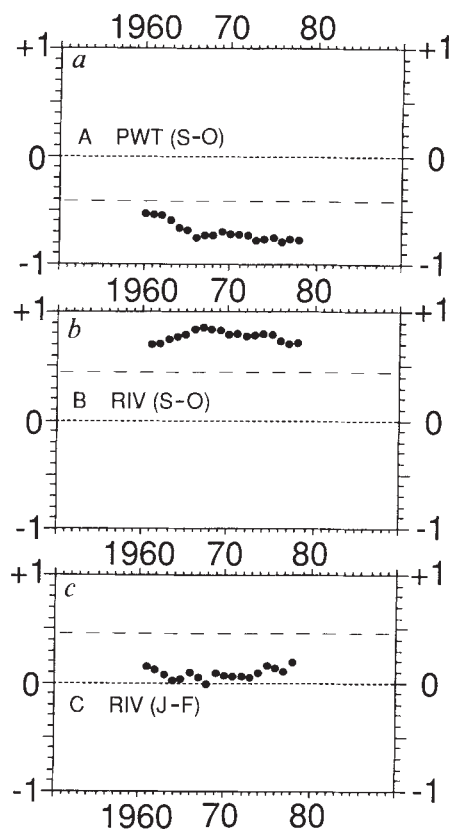


FIG. 3 Twenty one-year sliding mean correlations between January–February discharge and *a*, equatorial Pacific sea surface temperature anomaly in preceding September–October, PWT (S–O); *b*, river discharge in preceding September–October, RIV (S–O); *c*, river discharge in preceding January–February, RIV (J–F). Broken lines indicate, approximately, the 5% significance level.

varies more strongly than evapotranspiration from land surfaces, both in the annual cycle and from year to year. Accordingly, river discharge is primarily a proxy for rainfall, although the possible consequences of human activities should be considered. The discharge lagging behind rainfall (Fig. 2a, b) reflects a buffering effect resulting from the temporary storage in plant canopy and soil.

Water discharge varies considerably from year to year. Other diagnostic investigations (unpublished) indicate a high southern oscillation phase (defined by anomalously high/low pressure at Tahiti/Darwin) for summers with abundant river discharge, and accelerated cross-equatorial flow over the eastern Pacific, consistent with a northward displaced ITCZ. On the basis of these diagnostic studies, the following circulation indices were found to be particularly relevant with regard to anomalous river discharge in northern South America: a southern oscillation index, which is the pressure difference at Tahiti minus that at Darwin; an index, PWT (Fig. 1), of the sea surface temperature anomaly in the equatorial central Pacific (domain 2°–6° W, 90°–170° W; 2° N–6° S, 90°–180° W; 6°–10° S, 110°–150° W), which is also an index of the southern oscillation; the zonal wind component over the western Caribbean (domain 10°–20° N, 70°–80° W); and the meridional wind component over the eastern equatorial Pacific (domain 9°–6° N, 80°–90° W). Time series of these indices, as well as those of river discharge, were compiled for bi-monthly seasons.

Correlations between the discharge in January–February, the time of year of low stand, and some of the aforementioned elements are depicted in Fig. 3; correlations with the discharge a year earlier (Fig. 3c) are very small, but the persistence of discharge from September–October to January–February is substantially larger (Fig. 3b). For comparison, Fig. 3a shows the sizeable correlations with the September–October sea surface temperature anomaly in the equatorial Pacific. In fact, PWT for September–October proved to be a more powerful predictor of river discharge for January–February than the river discharge of September–October.

I developed regression models⁷ for river discharge as regressand/predictand, using the southern oscillation index, PWT, the zonal wind component over the western Caribbean and the meridional component over the eastern equatorial

Pacific (all two seasons earlier) as input to the regressions. The record up to 1970 (in the case of Essequibo 1950–70) served as a 'dependent' data set for training period to develop the regression models. The record 1971–87 was reserved as an independent data set or prediction. Prediction was successful for most rivers/seasons. In some cases it was useful to include antecedent hydrological information with the aforementioned atmospheric–oceanic indices. Overall, anomalous discharge is most predictable for the time of year of low stand.

For the January–February discharge of the Essequibo, a regression/prediction model was developed using September–October PWT as sole predictor. The forecast performance in the independent data set 1971–87 is shown in Fig. 3. The model accomplished prediction of 74% of the interannual variance. This performance compares favourably with the most successful methods applied in other areas in the tropics¹. Inasmuch as PWT can now be created in near real-time, this method offers the prospect of climate prediction on an operational basis. □

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Quantification of changes in lakewater chemistry in response to acidic deposition

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DESPITE intensive research, regional changes in lakewater chemistry in response to acidic deposition have not been adequately quantified. Palaeolimnology offers an independent means of testing the fundamental assumptions of acidification theory, but has not previously been used for regional assessment. Here we present rigorous documentation and quantification of regional lakewater acidification in the Adirondack Mountains of New York. The changes in acid-neutralizing capacity and pH that can be inferred from our palaeolimnological reconstructions indicate widespread recent acidification, but the changes are smaller than previously

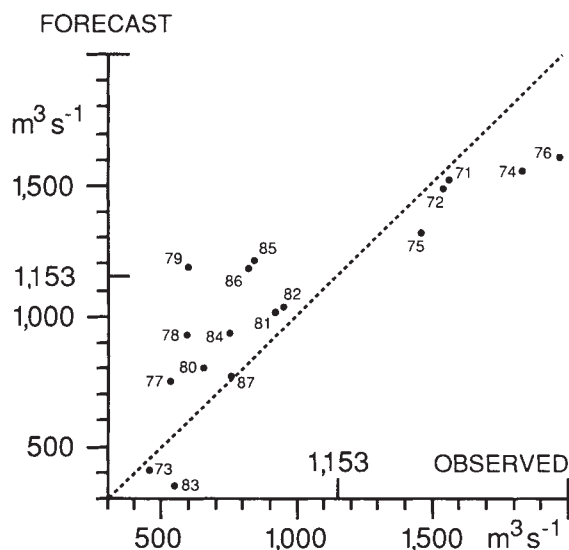


FIG. 4 Scatter diagram of forecast versus observed January–February discharge ($\text{m}^3 \text{s}^{-1}$). Regression base period (September–October equatorial Pacific sea surface temperature anomaly, PWT) is 1950–70 (mean $1,153 \text{ m}^3 \text{s}^{-1}$) and forecast period 1971–87. Numbers indicate the years and dotted line 45° angle. Correlation coefficient, +0.86. It is significant at the 1% level.

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estimated using empirical models, and suggest that the role of watershed and/or in-lake neutralization of acidic input is more important than previously assumed^{1,2}. Model-based estimates of future chemical change and consequent biological responses should therefore be re-evaluated in the light of these results.

The Adirondack Mountains of New York constitute the region of the United States that has been most intensively studied with respect to the acidity of surface waters and potential acidic deposition effects³⁻¹⁰. The Adirondacks receive high levels of atmospheric deposition of sulphur and nitrogen¹¹, and the sulphate in lake water is largely attributable to atmospheric deposition¹²⁻¹⁴.

Quantitative evaluations of regional acidification of Adirondack lakes have been largely based on comparison of recent and historic lakewater chemistry data^{8,9}. These comparisons consistently indicate recent acidification of some lakes, but differ in their estimates of the change that has occurred. Problems include a lack of documentation of historical sampling and analytical procedures, use of soft-glass containers that could have contributed alkalinity to stored samples, and uncertainties associated with the natural temporal variability of lakewater chemistry^{8,15,16}. It is therefore difficult to draw quantitative conclusions regarding historical acidification based on these data.

Palaeolimnological reconstructions of past lakewater chemistry are based on transfer functions derived from relationships between present chemistry and diatom fossil remains in lake surface sediments. Palaeolimnological data for inferring present-day and pre-industrial lakewater chemistry were collected as part of the PIRLA-II (Palaeoecological Investigation of Recent Lake Acidification) project¹⁷ from 36 Adirondack lakes that were included in the statistical sampling of the United States EPA Direct/Delayed Response Project (DDRP)¹⁸, a subset of the US EPA Eastern Lake Survey—Phase I (ELS-I)^{19,20}. The tops (present estimates, 0–1 cm depth) and bottoms (pre-1850 estimates, more than 30-cm depth) of the sediment cores were analysed for diatom fossil remains. Palaeolimnological data were included for 12 other Adirondack lakes sampled as part of PIRLA-I (ref. 21). Transfer functions were developed for inferring pre-industrial and present pH, Gran acid-neutralizing capacity (ANC_G) and dissolved organic carbon (DOC), and applied to data from the sediment cores. Background information, standardized palaeolimnological protocols, reproducibility, and quality assurance/quality control (QA/QC) guidelines for PIRLA-I and PIRLA-II have been described²²⁻²⁵. Measurements of present water chemistry were derived primarily from ELS-I (refs 19, 20), although chemical data for four lakes were taken from other surveys^{10,21}.

Although historical changes in acid-neutralizing capacity (ANC) and pH were estimated directly from palaeolimnological reconstructions, change in labile monomeric Al (Al_i) was estimated by a combination of palaeolimnological and empirical data. It is generally accepted that the labile fraction contains the Al species most toxic to fish. Aluminium concentration ($[\text{Al}_i]$) was measured in the ELS-II study, and for drainage lakes showed a linear relationship with H^+ during the autumn. The equation ($P \leq 0.0001$) and standard errors are given by $\text{Al}_i = 0.41(0.02) \times [\text{H}^+] + 0.75(0.26)$; $n = 33$; and $r^2 = 0.92$, where units are in μM . This relationship was used to estimate current $[\text{Al}_i]$ for 15 drainage lakes that were not included in ELS-II, and pre-1850 $[\text{Al}_i]$ for all drainage lakes, based on diatom inferred pre-1850 H^+ (from pH). The concentration of Al_i for two lakes was taken from ref. 7 and for a third lake was estimated from total monomeric Al and DOC¹⁴. Seepage and spring lakes generally have lower $[\text{Al}_i]$ than drainage lakes because their hydrological input excludes substantial surface water run off, and watershed contributions would mainly be highly neutralized groundwater inputs. Pre-industrial $[\text{Al}_i]$ for these lakes was therefore estimated as zero. We used the computer program ALCHEMI²⁶ for aluminium speciation calculations. The

equivalence of Al_i was calculated as the equivalent sum of aqueous free Al (Al^{3+}) and its hydroxide, fluoride and sulphate complexes.

Change in lakewater chemistry is presented as the difference in pH and ANC, inferred from diatoms, between the tops (present conditions) and bottoms (pre-1850) of the sediment cores. The pH calibration equation included 71 lakes ($r^2 = 0.91$, standard error (s.e.) = 0.28 pH units). Two ANC inference equations were derived, the primary one for lakes having $\text{ANC} \leq 100 \mu\text{eq l}^{-1}$ ($n = 56$, $r^2 = 0.86$, s.e. = $12 \mu\text{eq l}^{-1}$), and a second for higher ANC lakes ($> 100 \mu\text{eq l}^{-1}$) which had a larger standard error ($38 \mu\text{eq l}^{-1}$). Inferred pre-industrial $[\text{Al}_i]$ was low for most lakes; only 3 of 48 were estimated to have contained $> 6 \mu\text{eq l}^{-1}$ of Al_i . In the absence of mineral acidity before the onset of acidic deposition, most Al associated with organic acidity would probably have been organically complexed²⁷. Inferred changes in DOC were small and less than the standard error of the inference equation ($55 \mu\text{M}$) for most lakes. Associated small changes in organic anion concentrations (~ 2 – $4 \mu\text{eq l}^{-1}$) would have minimal effect on inferred base cation neutralization and are omitted in the calculations that follow.

Because the palaeolimnological estimates of historical ANC change (ΔANC) were calibrated to Gran titration ANC^{28} and the changes in DOC were inferred to have been very small, the palaeolimnological estimates of ΔANC_G correspond roughly to change in $[\text{HCO}_3^- - \text{H}^+]$. Aluminium change must be evaluated separately, in addition to ΔANC_G , to assess biologically relevant changes in water chemistry²⁹. Neglecting the small changes in organic acids, $[\Delta\text{ANC}_G - \Delta\text{Al}_i]$ is roughly equivalent to ΔANC , where ANC is defined as either $[\text{C}_B - \text{C}_A]$ or $[\text{HCO}_3^- - \text{H}^+ - \text{Al}^{n+}]$ (see ref. 30 for example), where C_B is the base cation sum ($\text{Ca}^{2+} + \text{Mg}^{2+} + \text{Na}^+ + \text{K}^+ + \text{NH}_4^+$) and C_A is the mineral acid anion sum ($\text{SO}_4^{2-} + \text{NO}_3^- + \text{Cl}^- + \text{F}^-$).

Inferred acidification (decreased $[\text{ANC}_G - \text{Al}_i]$) was generally greater for highly acidic lakes ($\text{ANC} \ll 0$), except for one bog-influenced seepage lake with a present ANC_G of $-80 \mu\text{eq l}^{-1}$ (Fig. 1). Acidification was limited to lakes having $\text{ANC}_G \leq 50 \mu\text{eq l}^{-1}$ (Fig. 1). Population estimates of historical change in lakewater chemistry for Adirondack lakes were generated based

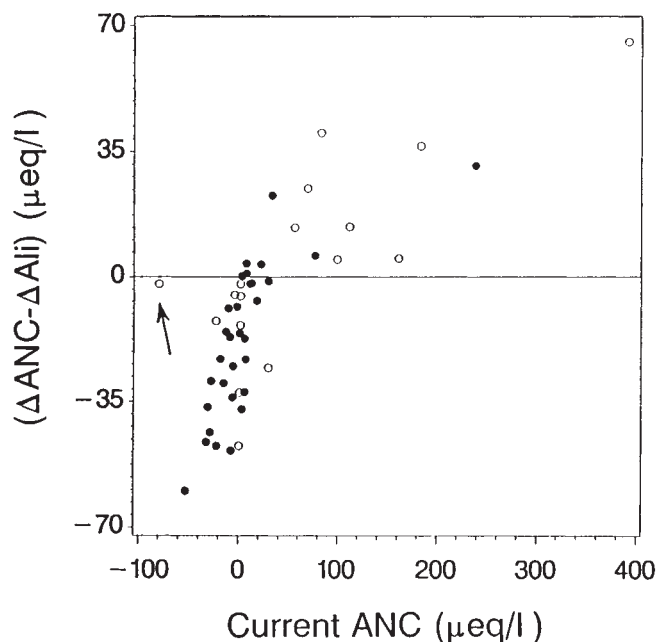


FIG. 1 Palaeolimnological inference of change in ANC, defined as $[\text{ANC}_G - \text{Al}_i]$, versus current ANC_G for 48 lakes in the Adirondack Mountains. Lakes identified as having watershed disturbances are coded by open circles. The far left outlier (arrow) is a bog influenced seepage lake.

on lakes in the statistical sampling. The median (and quartiles) inferred loss of ANC since pre-industrial times was -18 (-5 , -23) $\mu\text{eq l}^{-1}$ for acidic lakes, and -7 (0 , -13) for lakes that currently have ANC between 0 and $25 \mu\text{eq l}^{-1}$. For acidic lakes, inferred changes in Al_i were as large as changes in ANC_G . Acidified lakes were located primarily at elevations >600 m in portions of the Adirondack Park that receive the highest SO_4^{2-} loadings and annual precipitation $>110 \text{ cm yr}^{-1}$ (Fig. 2). Lakes that experienced historical increases in $[\text{ANC}_G - \text{Al}_i]$ tended to be located at lower elevations in areas receiving $<100 \text{ cm yr}^{-1}$ of precipitation (Fig. 2), and many showed evidence of watershed disturbance.

These estimates of historical acidification provide important information regarding catchment and surface-water response to acidic deposition. Empirical models for quantifying acidification are based on a charge-balance definition of ANC, assuming that some cations increased in response to mineral acid anion additions and/or the HCO_3^- anion decreased, to maintain electroneutrality^{1,2,31,34}. Henriksen^{31,32} initially assumed that only H^+ , Al^{n+} and HCO_3^- changed in response to increased $[\text{SO}_4^{2-}]$ but evidence was later presented for Norwegian³³ and North American¹ lakes indicating that increased base cation release (primarily $[\text{Ca}^{2+} + \text{Mg}^{2+}]$) accounted for up to 40% of added $[\text{SO}_4^{2-}]$, whereas from 60% to 100% of $[\text{SO}_4^{2-}]$ input reduced ANC (expressed as $[\text{HCO}_3^- - \text{H}^+ - \text{Al}^{n+}]$). The proportional change in base cations relative to the change in $[\text{SO}_4^{2-}]$ is referred to as the F -factor,

$$F = \Delta C_B / \Delta \text{SO}_4^{2-} \quad (1)$$

Specification of the appropriate F -factor determines the extent of acid neutralization and is the most important determinant of empirical model results. If $[\text{NH}_4^+]$ and $[\text{NO}_3^-]$ inputs are similar, the effects of nitrogen deposition can be considered by adding NO_3^- to the denominator in equation (1).

Our inferences of historical change in $[\text{ANC}_G - \text{Al}_i]$, based upon palaeolimnological reconstructions, were used to specify a distribution of F -factors for Adirondack lakes. For low ANC lakes ($<25 \mu\text{eq l}^{-1}$), we assumed a background $[\text{SO}_4^{2-}]$ of $20 \mu\text{eq l}^{-1}$, in agreement with other studies^{14,35}. Although background $[\text{SO}_4^{2-}]$ is likely to have been higher in some lakes that have $\text{ANC} > 25 \mu\text{eq l}^{-1}$, this is not relevant to our calculations, because the F -factors for these lakes were ≥ 1.0 , regardless. Furthermore, the calculations were not very sensitive to choice of background $[\text{SO}_4^{2-}]$ for the low ANC systems because only a small percentage of the estimated change in $[\text{SO}_4^{2-}]$ has resulted in a change in ANC.

Background $[\text{NO}_3^-]$ was assumed to be zero in the autumn, and was included in the model formulation because $[\text{NO}_3^-]$ is significant at this time in some of the Adirondack lakes (maximum, $18 \mu\text{eq l}^{-1}$). Thus,

$$\Delta \text{ANC}_G = \Delta C_B^* + \Delta \text{Al}_i - (\Delta \text{SO}_4^* + \Delta \text{NO}_3^-) \\ = F \times (\Delta \text{SO}_4^* + \Delta \text{NO}_3^-) + \Delta \text{Al}_i - (\Delta \text{SO}_4^* + \Delta \text{NO}_3^-) \quad (2)$$

and

$$F = 1 + [\Delta \text{ANC}_G / (\Delta \text{SO}_4^* + \Delta \text{NO}_3^-)] - [\Delta \text{Al}_i / (\Delta \text{SO}_4^* + \Delta \text{NO}_3^-)] \quad (3)$$

where the asterisk indicates correction for Cl^- and base cations charge balanced by Cl^- .

F -factors are generally considered to be less than 0.4 for acid-sensitive systems. But our calculations using equation (3) indicate a range from 0.5 to above 1.0 , and for most acidic lakes the F -factor was in the range 0.6 to 0.9 , illustrating the importance of watershed and/or in-lake processes in neutralizing acidic atmospheric inputs. The calculated F -factors increased with decreasing C_A^*/C_B^* (Fig. 3). The equation ($P \leq 0.0001$) and standard errors are given by $F = 1.17(0.05) - 0.29(0.05) \times [C_A^*/C_B^*]$. The inferred F -factor distribution decreased below 1.0 at C_A^*/C_B^* values ≥ 0.6 .

We concluded that 7 of the 48 Adirondack lakes in our data set had been acidic ($\text{ANC} \leq 0$) in pre-industrial times. Three of these are small (<4 hectare) seepage lakes, with relatively low $[\text{SO}_4^{2-}]$ (55 – $68 \mu\text{eq l}^{-1}$), $C_B \approx C_A$, and variable (251 – $1,189 \mu\text{M}$) DOC concentrations. Because of low base cation concentrations in these lakes, naturally occurring organic acids were apparently enough without acidic deposition to lower their pH (to 4.6 – 5.1). The other four lakes are high-elevation (>570 m), mostly small drainage lakes with relatively low $[C_B]$ (70 – $119 \mu\text{eq l}^{-1}$) and moderate DOC. They probably had ANC near zero before the onset of acidic deposition. Inferred pre-industrial values for these lakes were -1 to $-7 \mu\text{eq l}^{-1}$ of ANC and pH of 4.9 to 5.2 . Three were included in the DDRP statistical sampling, representing 3% of the ELS-I Adirondack lakes.

The median proportional changes in bicarbonate ANC (expressed as $[\text{HCO}_3^- - \text{H}^+]$), C_B^* and $[\text{Al}_i]$, relative to the estimated increase in $[\text{SO}_4^{2-} + \text{NO}_3^-]$ from pre-1850 to the present

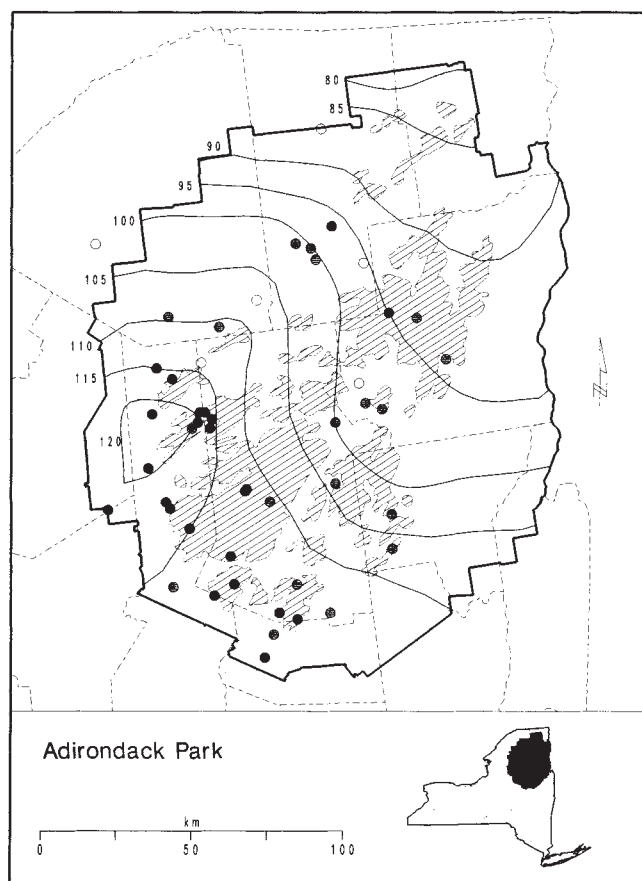
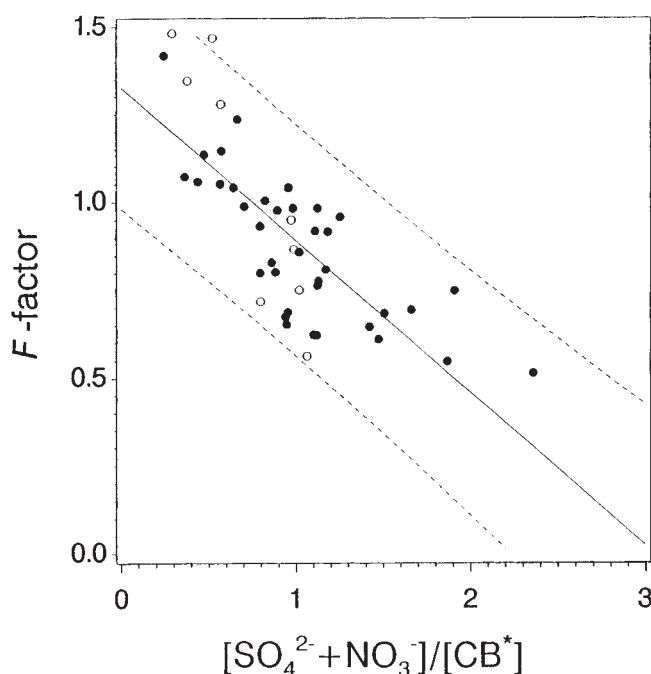


FIG. 2 Map of Adirondack Park showing location of palaeolimnological study lakes and precipitation contours (interval 5 cm yr^{-1}). Lakes are coded by inferred change in $[\text{ANC}_G - \text{Al}_i]$ ($\mu\text{eq per litre}$) from pre-1850 to the present: open circles, increase of 10 or more; shaded circles, -10 to 10 ; filled circles, decrease of -10 or more. High-elevation areas (>600 m) are shaded with cross-hatching.

FIG. 3 F -factors, calculated using equation (3) versus C_A^*/C_B^* for Adirondack lakes. C_A and C_B were corrected for measured $[Cl^-]$ to eliminate potential road salt contamination effects. The regression relationship and 95% confidence bounds are also presented. Lakes identified as having watershed disturbances are coded by open circles.



for low ANC ($\leq 25 \mu\text{eq l}^{-1}$) Adirondack lakes, were -0.11 , $+0.84$ and $+0.05$, respectively. These estimates agreed well with estimates of change based on the slopes of empirical distributions of present lakewater chemistry throughout the northeastern United States. The slopes ($\pm$ s.e.) of the relationships between $[HCO_3^- - H^+]$, $[Ca^{*} + Mg^{*}]$ and $[Al_i]$ versus $[SO_4^{*} + NO_3^-]$ for ELS-1 northeastern low ANC ($\leq 25 \mu\text{eq l}^{-1}$) lakes were -0.13 (± 0.04), 0.54 (± 0.9) and 0.07 (± 0.01), respectively. For northeastern lakes with present ANC $\leq 200 \mu\text{eq l}^{-1}$, the slope of $[Ca^{*} + Mg^{*}]$ versus $[SO_4^{*} + NO_3^-]$ was close to 1.0 (0.93 , s.e. = 0.08); no significant relationship with $[SO_4^{*} + NO_3^-]$ was observed at $P < 0.1$ for $[HCO_3^- - H^+]$; for $[Al_i]$, the slope was significant ($P \leq 0.0001$), but very small (0.01 , s.e. = 0.0003). This regional analysis assumes that a regression derived from spatial data can be used to predict trends in time, and thus that the distribution across space was initially homogeneous. Although such an assumption is difficult to substantiate, the agreement with palaeolimnological data is striking.

Thus, results of our palaeolimnological assessment of historical Adirondack lake acidification are in agreement with empirical distributions of present chemistry (space-for-time substitution). These results verify that many lakes in the Adirondacks have recently acidified as a result of increased C_A^*/C_B^* . The extent of acidification has been substantially less than hitherto believed, and the increase in $[Al_i]$ has accounted for a larger proportion of the acidification than was previously estimated for the Adirondacks^{1,2}. Classification of surface waters in North America as 'sensitive', based on ANC $\leq 200 \mu\text{eq l}^{-1}$ (refs 2, 36, 37), seems not to be sufficiently restrictive, given the present rates of acidic deposition. Based on our results in the Adirondack Mountains, a reasonable criterion for sensitivity to chronic lakewater acidification would be an ANC of $50 \mu\text{eq l}^{-1}$, although acidification may occur in surface waters having higher ANC values during spring and during periods of high discharge.

These results document widespread acidification of low-ANC Adirondack lakes, but the small magnitude of inferred acidification has important implications for the estimation of future response of lakewater chemistry to changes in acidic deposition. Apparently, lake chemistry has been less responsive to acidic atmospheric inputs, and watershed and/or in-lake neutralization processes have been of greater importance, than was generally assumed. Model-based estimates of future chemical change and consequent biological responses should

be re-evaluated in light of these historical-acidification estimates. □

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Preferential water leakage from fluid inclusions by means of mobile dislocations

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THE fluid density, carbon dioxide content, and therefore the pressure-temperature conditions derived from fluid inclusions in metamorphic rocks are commonly inconsistent with coexisting mineral assemblages. Here we report hydrothermal experiments mimicking isobaric cooling and isothermal decompression, which indicate changes in density and composition without significant change in volume of H₂O–CO₂ inclusions in quartz. A simultaneous change to CO₂-rich compositions and lower densities indicates that water is leaking from the inclusions. We argue that preferential water leakage is made possible by microscopic unmixing of the H₂O–CO₂ mixture caused by physical and chemical interactions with the inclusion wall. Water transport then occurs by the movement of water-saturated dislocations through the hydrothermally recrystallizing quartz.

Significant discrepancies often exist both between the measured densities of gas-rich fluid inclusions and those calculated from metamorphic pressure-temperature conditions as inferred from mineral assemblages, and between observed and calculated values of water content in fluid inclusions for appropriate mineral assemblages (for example, studies on Rogaland, Norway^{1,2} and Naxos, Greece^{3–6}). The discrepancies have led to suggestions concerning re-equilibration of fluid inclusions during post-peak deformation, for example by dynamic recrystallization^{7–9}. As such, deformation during isobaric cooling (for Rogaland²) or isothermal decompression by syn-metamorphic tectonic extension (for Naxos¹⁰) have been proposed.

Experimental re-equilibration of fluid inclusions resulting only in density changes, has been achieved by (1) volume changes without significant diffusion of H₂O through quartz¹¹; (2) decrepitation^{12–16}, and (3) diffusion of H₂O through the quartz lattice^{14,15} as point defects (interstitial molecules^{17,18} and substitutes^{19,20}) or as water-related species through dislocation lines^{16,21–23}. Depending on temperature and crystallographic orientation with respect to deviatoric stress state, H₂O may diffuse through dislocation lines due to activation of glide systems. Until now, no compositional change of H₂O–CO₂ fluid inclusions has been experimentally studied by hydrothermal re-equilibration²⁴.

In this study, fluid inclusions were synthesized at 200 MPa and 835 K for 38 days according to the method of Sterner and Bodnar²⁵ in cores drilled out of optically clear Brazilian quartz. The fluid composition was 79 ± 3 mol% H₂O and 21 ± 3 mol% CO₂ with no addition of salt, hydroxide or acid, in order to avoid rapid quartz recrystallization and incorporation of imperfections. Flat fluid inclusions were selected from a three-dimensional inspection with a U-stage to characterize their

morphology and volume percentages of the gas species (Fig. 1). A microthermometric study yielded an average homogenization temperature (T_H) of the CO₂ phases at 304.2 K and of the total H₂O–CO₂ content at 574.4 K (Table 1). Electron microscopy (TEM) was performed to identify defect structures in the healed crack. The thin foils were prepared from quartz cores from duplicate experiments in order to keep the core, intended for further re-equilibration, undamaged (see below).

Isobaric cooling was experimentally reproduced by a second anneal at 673 K and 200 MPa for 21 days (Fig. 2). A pure H₂O environment of the quartz core was chosen to distinguish newly formed fluid inclusions from the original CO₂-bearing fluid inclusions from the first run. The identical individual fluid inclusions were again photographed (Fig. 1b) and T_H was remeasured (Table 1). Comparison of morphologies of these flat fluid inclusions assures that the volumes changed less than 4 vol% and presumably, this may hold for all fluid inclusions. The quartz was re-examined by TEM after microthermometry. The foils were made of small, 0.5-mm-thick slices of the same core used in both synthesis and re-equilibration experiments. Direct comparison of the dislocations before and after re-equilibration was impossible. As result of the second run, a few pure H₂O fluid inclusions were synthesized in cracks, which were not completely annealed after the first run. Their average density of 0.0445 mol cm⁻³ reflects the new conditions. The identical H₂O–CO₂ fluid inclusions from the first run now contained lower densities than after the first run as illustrated by volume increases of the vapour and liquid CO₂ bubbles at the expense of H₂O volume (Fig. 1a, b). The T_H of the CO₂ phases is now in the range 266.0–304.1 K, whereas T_H of the total content is in the range 541.0–595.4 K (Table 1).

Water content was calculated by density measurements and volume estimations of the fluid species based on surface measurements. A few fluid inclusions retained their original water content values, but most were lower than the original values. Any volume adjustments and infiltration of H₂O would result in lower T_H (Fig. 2). Seemingly contradictory, preferential leakage of H₂O is responsible for instantaneously higher T_H and simultaneous internal pressure drop. Fugacity-, chemical- and pressure-gradients are considered as driving forces, resulting only in infiltration of H₂O. Water leaked out of the fluid inclusions, so the gradients cannot be the direct driving force for outward H₂O diffusion and the diffusion cannot occur by channelized cracks or pores. The available data for point-defect diffusion in quartz^{26–29} prove that this mechanism is too slow to be effective within the time of experimentation.

Dislocations in hydrothermal quartz were imaged with TEM. Most of the apparently isolated inclusions end in dislocation arrays (Burgers vectors parallel to $\langle a \rangle$) were determined by tilting experiments) or larger channel-like imperfections. Dislocation arrays seem to be pinned on fluid inclusions (Fig. 3). The formation of bubbles on dislocation lines^{20,30,31} after heat treatment and numerous secondary inclusions^{12,15} ('implosion haloes'¹¹) surrounding the central inclusion, which do not show signs of micro-cracking, argue for H₂O transport by dislocation lines or larger imperfections.

Over- and under-pressures of the fluid inclusions are supposed to constitute a driving force for the development of dislocations. Mobile dislocations are activated by a deviatoric stress state around the fluid inclusions due to the lattice discontinuity and their mobility may result in an episodic tapping of fluid inclusions. The dislocations may become saturated with water from the fluid inclusions. The dislocation loops, which move from the interior of the crystal to the surface, can be considered as 'water-buckets', able to operate in both internal over- and under-pressure conditions. Because of the high length:diameter ratio, the volume of H₂O transport may be larger than the claimed volume decrease of fluid inclusions by pore collapse. Also, fluid inclusions are principally major imperfections and they may act as sources of dislocation multiplication. The

TABLE 1 Duration of the experiment, range of homogenization temperatures (K) of CO₂ and CO₂-H₂O mixtures of the original and new experiments

	Duration (in days)	Homogenization CO ₂ phases			Homogenization H ₂ O-CO ₂ phases		
		$T_{\min}$	$T_{\max}$	T_{av}	$T_{\min}$	$T_{\max}$	T_{av}
Original experiment	38	303.5*	304.4*	304.2*	573.1	575.7	574.4
New experiment	21	266.0	304.1	—	541.0	595.4	—

There were 57 measurements per experiment.

* Temperatures of inclusions that homogenize in the liquid phase (others homogenize in the gas phase). The original fluid inclusions yield a narrow range of both homogenization temperatures, whereas the recrystallized fluid inclusions in the new experiment display a broad range.

mechanism does not explain preferential leakage of water from a single-phase CO₂-H₂O mixture in fluid inclusions during experimentation. Local unmixing on near-molecular scale must occur for the preferential leakage. This may be caused by the following: (1) The physical and chemical surface interaction between hydrophilic quartz and water dipoles. The surface of the fluid inclusions, the channel-like imperfections and the dislocation lines may be wetted by a molecular film of H₂O attached on interfacial silanol groups. On a larger scale, similar surface interactions, which may prevent CO₂ molecules from escaping through dislocations, have been proposed for fluid wetting of microcracks and grain boundaries in rocks³². Fluids with high CO₂ content ratios form isolated drops at grain corners, whereas at high H₂O contents a three-dimensional continuity of a fluid film exists. (2) The restriction of the dislocation size^{33,34}. The dislocations may be too small in diameter for effective molecular pipe-diffusion of CO₂ molecules, which are larger than H₂O molecules. (3) The formation of H₂CO₃

molecules, by the association of CO₂ and H₂O. These molecules influence the original fluid composition for small CO₂ contents. The dimensions of H₂CO₃ molecules are even larger than CO₂ molecules, enforcing argument (2).

We conclude that a wide range of fluid-inclusion compositions can be generated from one type of metamorphic fluid, by preferential H₂O leakage as a result of local variations in the degree of hydrothermal synmetamorphic recrystallization, even within a single trail. In experimental hydrothermal simulations of isobaric cooling (described here) and isothermal decompression³⁵ a decrease of density was caused by preferential leakage of water.

In theory, compositions of practically pure N₂-CH₄-CO₂ gaseous fluid inclusions are thermodynamically forbidden at high-grade peak metamorphic conditions (for Rogaland³⁶). The argued H₂O leakage out of originally water-rich fluid inclusions containing CH₄, CO₂ and N₂ may result in extremely gas-rich fluid inclusions.

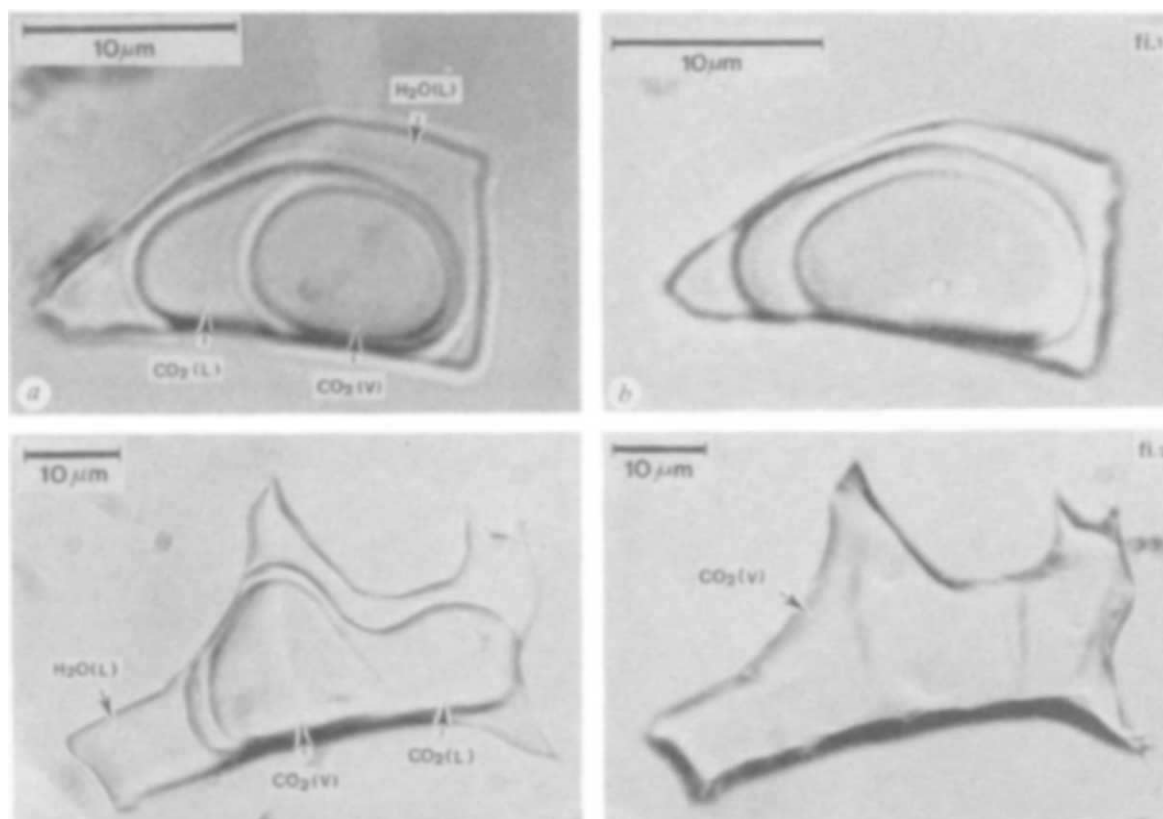


FIG. 1 *a*, Photomicrographs of three-phase fluid inclusions generated in the original experiment, with 21 mol% CO₂. At 293 K, the volume percentages of H₂O(l), CO₂(l) and CO₂(v) are 40, 39 and 21 respectively. Both inclusions homogenize at 304.3 K for the CO₂ and at 574.8 K for the CO₂-H₂O phases. *b*, Photomicrographs of the identical inclusions after the new experiment, simulating isobaric cooling. Only minor solution and precipitation is illustrated. At 293 K the volume percentages of H₂O(l), CO₂(l) and CO₂(v) have

been changed to 31, 29 and 40 respectively for fluid inclusion 1 (fi. 1) and to 10, 0 and 90 respectively for fluid inclusion 2 (fi. 2). The H₂O(l) phase of fi. 2 is concentrated in the edges of the inclusion. The homogenization temperatures of the CO₂ and the CO₂-H₂O phases for fi. 1 have been changed to 303.6 and 579.5 K, respectively and for fi. 2 to 269.8 K and 552.8 K. The calculated mol% CO₂ for fi. 1 and fi. 2 are 26 and 46, respectively.

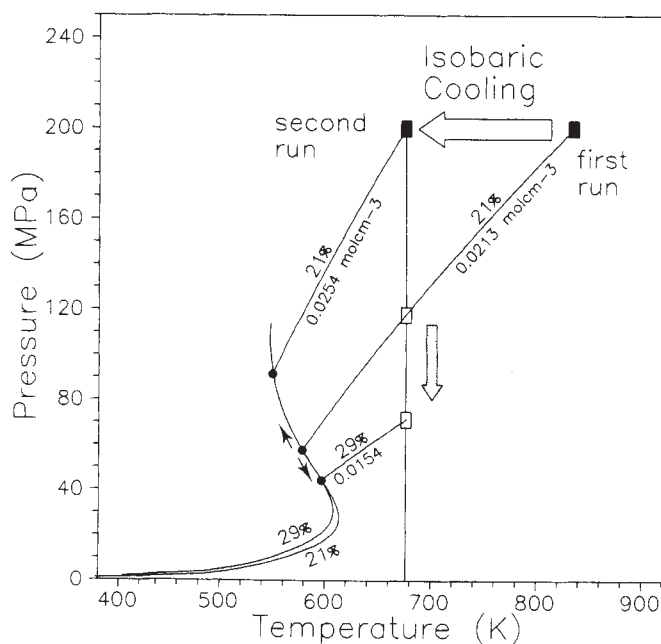


FIG. 2 Pressure-temperature diagrams display the experimental conditions (black squares). The curved lines are the projections of the experimentally determined H_2O - CO_2 mixing areas³⁷ for 21 and 29 mol% CO_2 . The corresponding isochores and their densities (mol cm^{-3}) are calculated as nearly straight lines with the MRK equation of state³⁸. Isobaric cooling is indicated with the large open arrow. The shaded arrow indicates the development of the underpressure in the fluid inclusions due to preferential leakage of H_2O at new experimental conditions (see text). The P_{intern} at the new conditions is indicated.

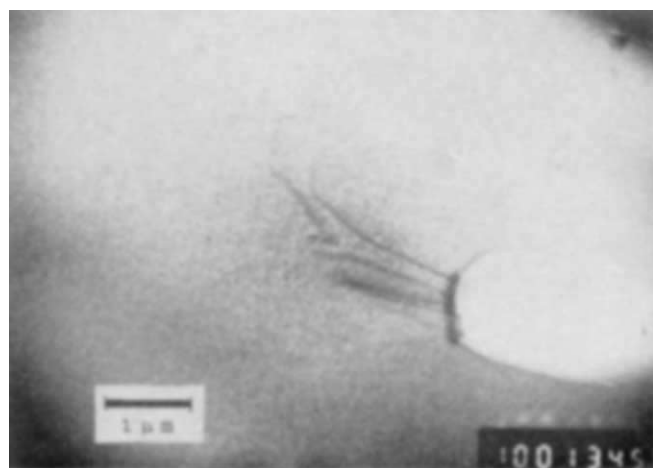


FIG. 3 The bright-field electron micrograph (TEM) of a synthetic fluid inclusion (white) produced in the original experiment illustrates a suite of six pinned dislocations.

The density of CO_2 -rich fluid inclusions in metamorphic quartz lenses (for Naxos^{3,4}) are adjusted to post-main metamorphic conditions by H_2O leakage and therefore, the CO_2 content is too high compared with the calculated CO_2 content derived from mineral equilibria. □

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Genetic diversity in Sargasso Sea bacterioplankton

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BACTERIOPLANKTON are recognized as important agents of biogeochemical change in marine ecosystems, yet relatively little is known about the species that make up these communities. Uncertainties about the genetic structure and diversity of natural bacterioplankton populations stem from the traditional difficulties associated with microbial cultivation techniques. Discrepancies between direct counts and plate counts are typically several orders of magnitude, raising doubts as to whether cultivated marine bacteria are actually representative of dominant planktonic species¹⁻³. We have phylogenetically analysed clone libraries of eubacterial 16S ribosomal RNA genes amplified from natural populations of Sargasso Sea picoplankton by the polymerase chain reaction⁴. The analysis indicates the presence of a novel microbial group, the SAR11 cluster, which appears to be a significant component of this oligotrophic bacterioplankton community. A second cluster of lineages related to the oxygenic phototrophs—cyanobacteria, prochlorophytes and chloroplasts—was also observed. However, none of the genes matched the small subunit rRNA sequences of cultivated marine cyanobacteria from similar habitats. The diversity of 16S rRNA genes observed within the clusters suggests that these bacterioplankton may be consortia of independent lineages sharing surprisingly distant common ancestors.

Our purpose was to identify genetic markers that could be used to examine the distributions and diversity of bacterioplankton with nucleic acid hybridization probes in routine studies.

The Sargasso Sea, a central oceanic gyre, was chosen as a sampling site because it provides an example of a microbial population adapted to life under extremely low nutrient conditions⁵.

Twelve cloned 16S rRNA genes chosen at random were sequenced. Two pairs of clones and one set of four clones were identical. Similarities among the aligned sequences indicated that the clones fell into two clusters (Fig. 1). All the unknown sequences conformed closely to secondary structure models⁶ for prokaryotic 16S rRNAs and showed no unusual base pairings or structural deviations in phylogenetically conserved regions, indicating that 'shuffle-gene' products were not present⁷ (Fig. 2). In each case the branches shown in Fig. 1 were found to result from changes either localized in variable regions of the molecule or verified by compensating base changes across helices.

Phylogenetic analysis showed that one cluster of sequences (the SAR7 cluster) branched within the radiation of oxygenic phototrophs, which includes cyanobacteria, chloroplasts and prochlorophytes (ref. 8 and Fig. 3). Similarities among the unknown sequences of the SAR7 cluster ranged from 0.952 to 0.978 for the positions indicated in Fig. 2. Comparisons with 16S rRNA gene sequences from four unicellular marine cyanobacteria (WH7202, WH7805, WH8103, WH8501; D. L. Distel and J. B. Waterbury, unpublished data)⁹ yielded similarity values ranging from 0.801 to 0.965, but no exact matches with the unknowns. The highest similarity observed between the unknown and known sequences was for the SAR7 and WH8103

comparison. Strain WH7805, which lacks phycoerythrin pigments, and strain WH8103, which contains phycoerythrins and is motile, are representative of cultivated cyanobacterial picoplankton¹⁰. Thus, the data indicate a high diversity of 16S rRNA gene lineages among closely related marine cyanobacteria, and suggest at least three novel lineages in the Sargasso Sea.

Eight clones belonging to the second gene cluster (SAR11 cluster) were sequenced. Comparisons to a database of more than 200 known eubacterial 16S rRNA sequences indicated that the unknown organisms were most similar to members of the α -purple eubacterial phylum¹¹. The highest similarity observed, however, 0.82 to *Agrobacterium tumefaciens*, was low in contrast to typical eubacterial inter-phylum relationships, which vary from 0.75 to 0.83 for the same coding region⁸. This suggests that the SAR11 cluster is a novel, deep phylogenetic branch of the eubacteria. Furthermore, these organisms are clearly distinct from the major genera of cultivated heterotrophic marine bacteria, including the common genera *Oceanospirillum*, *Vibrio*, *Photobacterium* and *Alteromonas*, which are members of the γ -purple phylum¹².

We sought to verify the significance of the SAR11 genotype by hybridizing an oligonucleotide probe complementary to con-

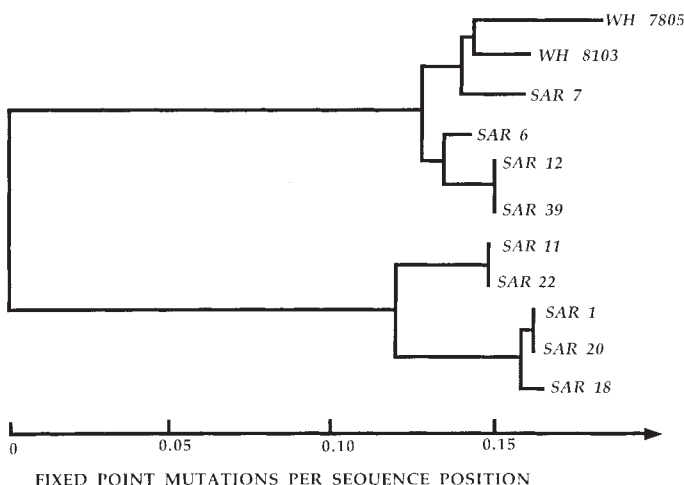


FIG. 1 Unrooted phylogenetic tree depicting relationships among Sargasso Sea bacterioplankton 16S rRNAs. The analysis includes the 16S rRNA sequences of two cultivated strains of marine cyanobacteria, WH8103 and WH7805. The sequences of clones SAR67 and SAR78 were identical to those of SAR1 and SAR20; SAR77 differed at one position (not shown). A total of 230 positions located at the 5' region of the gene, including both conserved and hypervariable domains, were included within the analysis (*E. coli* positions 101–344). Phylogenetic trees were constructed by a distance matrix method^{23,24,25}.

METHODS. The picoplankton samples were collected in April by tangential flow filtration on Durapore 0.1 μ m fluorocarbon membranes from a depth of 1–2 m at hydrostation S (32°4' N 64°23' W). The small subunit ribosomal RNA genes were amplified from bulk picoplankton DNA by a modification of the polymerase chain reaction²⁶. The reaction conditions were: 1 μ g template DNA; 2' at 94 °C, 2' at 37 °C, 7' at 72 °C; 30 cycles. The amplified genes were cloned as *Bam*HI/*Pst*I fragments into M13 phage mp18 and sequenced twice, once with inosine substituting for guanosine, using the dideoxy chain termination method²². The amplification primers (OX1 and OX2) were designed with a bias towards the cyanobacterial phylum of the eubacteria. Primers OX1 and OX2 are, respectively, 90% and 82% similar to eubacterial consensus sequences. The sequences of the amplification primers are: OX1, GTGTCGACAGAGAGTTTGATCCTGGCTAGG; OX2, CACGATCCAAGGAGGT-GATCCANCCNACC, where the domain complementary to the coding region is indicated in bold.

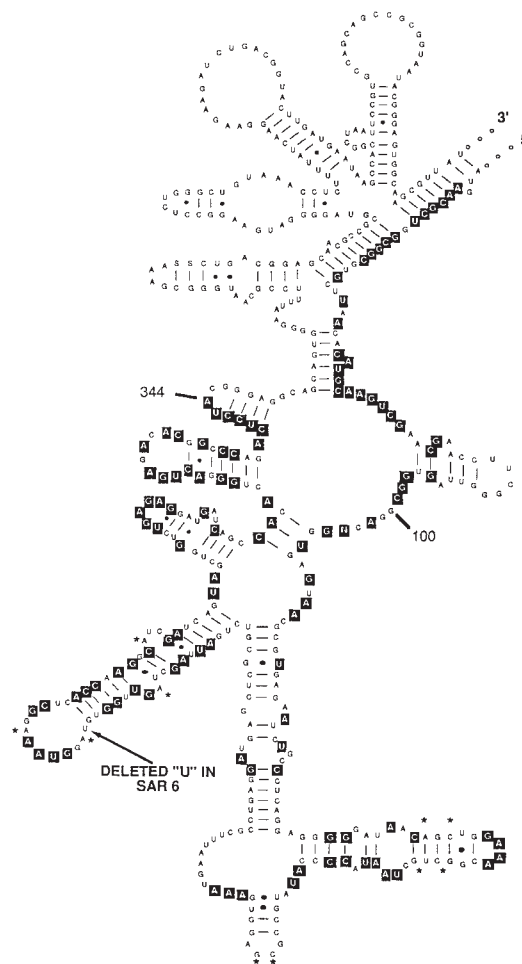


FIG. 2 Secondary structural model for 5' region of SAR7 cluster 16S rRNAs. Backshadowed characters indicate the eubacterial consensus sequence. The positions marked by asterisks are the sites of variations within the SAR7 cluster. The region used for the comparisons presented in Fig. 1 extends from position 101 to position 344. Numbering corresponds to the *E. coli* 16S rRNA structural model²⁷. A structural analysis of the complete genes indicated that the SAR6/SAR7 dichotomy (the deepest branch of the SAR7 cluster) was supported by seven pairs of compensating base changes across helices (data not shown).

served regions of the SAR11 16S rDNAs to ribosomal RNAs isolated from several natural microbial populations (Table 1). The amount of prokaryotic rRNA in the samples was determined by the hybridization of phylogenetic group-specific rRNA probes complementary to rRNAs from the primary kingdoms¹³. Ribosomal RNA from the SAR11 cluster accounted for 15% of prokaryotic RNA (12.5% of total RNA) in the Sargasso Sea, was detectable in coastal waters from Ferry Reach, Bermuda, and Key Biscayne, Florida, but was not detectable in water from Yaquina Head, Oregon. Because the specific RNA content of prokaryotic cells is proportional to growth rate, the rRNA of natural microbial populations provides a better estimate of metabolic activity than DNA, which could be derived from dormant organisms. The data suggest that the occurrence of the SAR11 cluster organisms may be restricted, possibly to tropical or oligotrophic oceanic regions.

Although there are no set rules for relating rRNA similarities to taxonomic units, the deepest branches within both clusters (0.96 for the SAR7 cluster; 0.94 for the SAR11 cluster) suggest at least 'species' level divergence. For the same positions represented in Fig. 1, *Escherichia coli* and *Salmonella enteritidis* have a similarity of 0.98. Among 11 strains representing 3 subspecies of *Lactococcus lactis*, 16S rRNA similarities of 0.97 to 0.99 are closely correlated with phenotypic differences (M. S. Salama, W. E. Sandine and S.J.G., unpublished results). But, the significance of the variation among the shallowest branches

TABLE 1 Analysis of microbial population rRNA by the hybridization of kingdom, universal, and SAR11 cluster oligodeoxynucleotide probes

	Population composition (%)			
	Eubacterial	Eukaryotic	Archaeobacterial	SAR11 cluster
Sargasso Sea	81.0	19.0	0.0	12.50
Ferry Reach	66.6	13.7	0.0	1.38
Key Biscayne	74.6	25.4	0.0	0.53
Yaquina Head	85.2	6.7	0.0	0.00

Binding of the phylogenetic group-specific probes was determined relative to universal probe binding using arrays of known RNAs (0, 2.5, 5, 10, 20, 30, 40, 50 ng) on nylon membranes¹³. DNA oligodeoxynucleotide probes were labelled with T4 polynucleotide kinase and γ -[³²P]ATP to a specific activity of between 1×10^8 – 2×10^8 c.p.m. μg^{-1} . The amount of bound probe was measured using a gas ionization scanner (Ambis systems). The compositions of the populations were determined from the binding of the kingdom-specific probes according to the following equation:

$$\%X = \frac{\delta C/\delta N \times (\delta U/\delta N)^{-1} - \sum_{i=1}^r [\delta R_i/\delta N \times (\delta U/\delta N)^{-1}]r^{-1}}{\sum_{i=1}^p [\delta P_i/\delta N \times (\delta U/\delta N)^{-1}]p^{-1} - \sum_{i=1}^r [\delta R_i/\delta N \times (\delta U/\delta N)^{-1}]r^{-1}} \times 100$$

where X , amount of specific RNA in an RNA sample; $\delta C/\delta N$, group-specific probe bound per unit of sample RNA; $\delta R_i/\delta N$, group-specific probe bound per unit of i th heterologous RNA standard; r , number of heterologous RNA standards; $\delta P_i/\delta N$, group-specific probe bound per unit of i th homologous RNA standard; p , number of homologous RNA standards; $\delta U/\delta N$, universal probe bound per unit of RNA (either sample or standard). The rRNA standards were: *Escherichia coli*, *Lactococcus lactis*, *Pseudomonas aeruginosa*, *Planctomyces maris*, *Halobacterium volcanii*, *Sulfolobus solfataricus*, *Strongylocentrotus purpuratus*, *Urechis caupo*, and *Lumbricus* sp. The seven oligonucleotide probes used were: universal probes, ACGGCGGTGTGTRC {1,406–1,392} and GWATTACCGCGGCKGCTG {536–519}; Eubacterial probe, CDCCGCTGTGCGGGCCC {944–927}; Eukaryotic probes, GGGCATCACAGACCTG {1,209–1,195} and GGTCCYTCTCCGGAATCGAACC {321–300}; Archaeobacterial probe, GCGCCTGTGTCSCCCCGTAGGCC {361–338}; and SAR11 cluster probe, AAGCTTTCTCGTAAGACTTAT {220–178}. The numbering corresponds to homologous positions in the *E. coli* 16S rRNA²². Linear regressions calculated by the least squares method were used to determine $\delta A/\delta N$, where A is C , R_i , P_i , or U from the standard arrays. The range between 0 and 50 ng RNA was found to be linear and was therefore used for all calculations of slope. The slopes for the two universal and eukaryotic probes were averaged. SAR11 cluster representation was determined as the ratio between the molar amounts of SAR11 cluster and universal probes bound because no positive rRNA controls are available for SAR11 organisms.

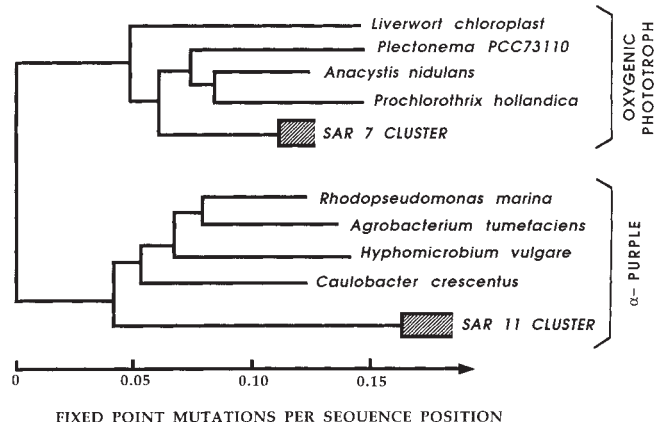


FIG. 3 Phylogenetic relationships of SAR7 and SAR11 16S rDNA sequence clusters to a collection of 16S rRNA sequences representing the oxygenic phototroph^{8,28} and α -purple eubacterial phyla²⁹. Four clones were sequenced completely (SAR6, SAR7, SAR1 and SAR11) and used for the inference of distant relationships. The tree was rooted using the sequences of *Bacillus subtilis* and *Heliobacterium chlorum*^{8,30}. The analysis was restricted to 900 sequence positions. Regions of uncertain homology between phyla, including hypervariable domains, were excluded from this analysis. Hence, the variability within the clusters (indicated by the hatched boxes) is about 0.01 similarity units less in this figure than in Fig. 1. The 3'-terminal domain of the 16S rRNAs was excluded from the analysis because of an internal *Bam*HI restriction site in clones SAR1 and SAR11 at position 1,190.

of the clusters (for example SAR1/SAR18) is uncertain. Some of the variability may be the result of microevolutionary divergence within multicopy rRNA gene families¹⁴. The location and pairing of the sequence changes within secondary structures suggests that errors and artefacts cannot account for a significant proportion of the observed variation^{15,16}. Furthermore, the highest error rate reported for Taq polymerase (1 in 400 after 30 amplification cycles) would result in an average similarity of 0.995 (ref. 4).

We speculate that some of the sequence diversity within the clusters may result from the essentially clonal nature of bacterial reproduction. Linkage disequilibria between allozyme loci in *E. coli* support the view that chromosomal recombination is low within eubacterial species, and that microbial populations are clonal¹⁷. High genetic diversity has been observed in *Pseudomonas cepacia*, and correlated with habitat variability, suggesting microgeographical adaptation as a result of selection¹⁸. For planktonic bacterial populations, viral infections may be a source of selective pressures contributing to the divergence of lineages¹⁹.

The results support the widespread view that microbial ecosystems contain novel, uncultivated species²⁰. Also, we have observed unexpected genetic variability within the clusters. The data imply that many closely related 16S rRNA lineages coexist within relatively well circumscribed microbial populations. Molecular phylogenies of cultivated bacteria have provided little insight into the genetic structure of natural microbial populations, largely because they have focused on strains with unique characteristics. The genetic diversity indicated by this study suggests that rRNAs may provide a tool for analysing prokaryotic population biology, as well as for identifying fundamentally unique community members, such as the SAR11 cluster²¹. □

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16S rRNA sequences reveal numerous uncultured microorganisms in a natural community

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MICROBIOLOGISTS have been constrained in their efforts to describe the compositions of natural microbial communities using traditional methods. Few microorganisms have sufficiently distinctive morphology to be recognized by microscopy. Culture-dependent methods are biased, as a microorganism can be cultivated only after its physiological niche is perceived and duplicated experimentally. It is therefore widely believed that fewer than 20% of the extant microorganisms have been discovered^{1,2}, and that culture methods are inadequate for studying microbial community composition³⁻⁷. In view of the physiological and phylogenetic diversity among microorganisms⁸, speculation that 80% or more of microbes remain undiscovered raises the question of how well we know the Earth's biota and its biochemical potential. We have performed a culture-independent analysis of the composition of a well-studied hot spring microbial community, using a common but distinctive cellular component, 16S ribosomal RNA. Our results confirm speculations about the diversity of uncultured microorganisms it contains.

We developed a cloning method for retrieving naturally occurring 16S rRNA sequences⁹ that is more selective than methods previously proposed for recovering 16S rRNA genes^{10,11}. The selectivity is based on the synthesis of complementary cDNA from 16S rRNA templates (termed 16S rcDNA) using an oligonucleotide primer complementary to a universally conserved region of 16S rRNA. Cloned 16S rcDNA sequences from the community are then compared with 16S rRNA sequences of microorganisms isolated from this or similar habitats.

We studied 16S rcDNA sequences from the 55 °C cyanobacterial mat of Octopus Spring, Yellowstone National Park. This totally microbial community should have a restricted¹² and relatively constant¹³ composition. It has been well-characterized with respect to component microorganisms by microscopic and culture methods¹³; a single cyanobacterium, *Synechococcus lividus*, and a photosynthetic bacterium, *Chloroflexus aurantiacus*, are thought to be responsible for production of the mat, and several bacteria which may participate in mat decomposition have been isolated. The mat has also been a rich source of phylogenetically interesting bacteria⁸. Studies of lipid cellular components have indicated, however, the presence of as yet undescribed community members^{14,15}.

Detailed analysis of primary and secondary structure provides convincing evidence that the cloned 16S rcDNA sequences correspond to 16S rRNA sequences. For example, Fig. 1 shows how primary sequence conservation and compensatory mutations in double-stranded regions accommodate the folding of a 16S rcDNA sequence recovered from the mat to match a secondary structure reported for the *Escherichia coli* 16S rRNA. Most of the sequence similarity data we present are from a conservative, restricted analysis which limits sequence comparison to regions in which nucleotides can be unambiguously aligned. This ensures that differences are not due to misalignment within highly variable sequence regions^{16,17}. The variable regions were included in unrestricted comparisons as a more rigorous test of identity between sequences.

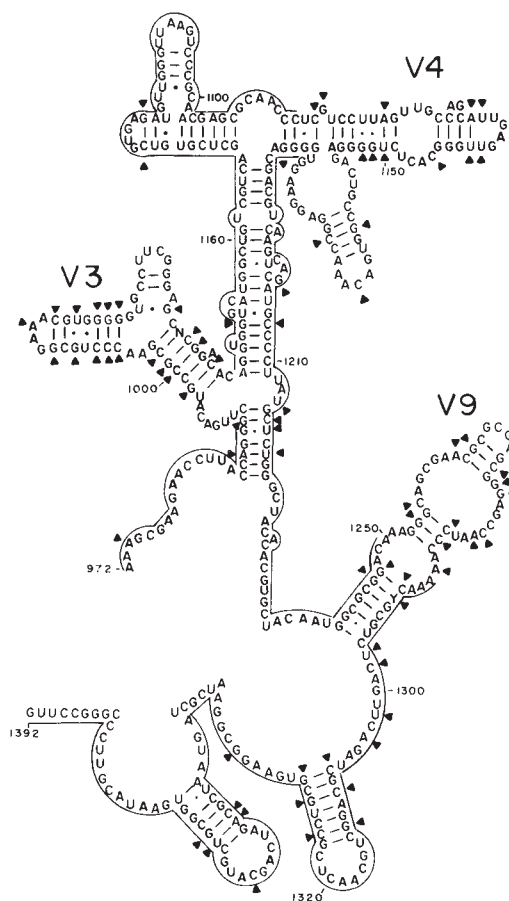


FIG. 1 Secondary structure of the most frequently observed Octopus Spring cyanobacterial mat 16S rcDNA sequence. The type-A sequence, (consensus of III-3, -5, -18, -21, and -22) was folded into the secondary structure reported for *E. coli* 16S rRNA²⁹. "▲" indicates a nucleotide different than that found at the same position in the *E. coli* sequence. The line indicates positions which were included in restricted sequence similarity analysis; variable sequence regions (V3, V4 and V9)³⁰ were excluded.

TABLE 1 Similarity between Octopus Spring cyanobacterial mat 16S rcDNA sequences

Sequence type	Number of nucleotides	16S rcDNA	III-3	III-5	III-18	III-21	III-22	Percent similarity with 16S rcDNA*						
A	397	III-3	—	100.0	100.0	100.0	100.0	97.3						
A	205	III-5	100.0	—	100.0	100.0	100.0	97.5						
A	424	III-18	100.0	100.0	—	100.0	100.0	97.0						
A	413	III-21	100.0	100.0	100.0	—	100.0	97.0						
A	415	III-22	100.0	100.0	100.0	100.0	—	97.1						
B	390	II-18	98.7	98.1	98.3	98.3	98.3	—						
C	164	III-15	84.6	84.7	84.8	84.8	84.7	84.9	—	100.0				
C	387	II-23	87.8	82.9	86.6	86.4	86.5	88.3	100.0	—				
D	118	II-13	86.7	86.7	86.8	87.6	87.5	87.5	74.3	74.0	—	100.0		
D	128	II-17	86.7	86.7	86.8	87.6	87.5	87.5	74.3	74.0	100.0	—		
E	376	II-2	89.2	84.9	88.1	88.4	88.9	89.0	80.3	89.3	84.9	84.9	—	
F	144	II-3	84.8	84.0	84.0	84.8	84.8	84.8	76.4	77.1	90.4	90.4	88.7	—
G	588	II-12	86.1	80.0	84.6	84.1	84.8	84.3	72.2	83.0	82.9	82.9	87.9	83.8
H	423	II-30	88.7	85.0	86.1	88.1	88.8	87.7	77.9	84.6	84.8	84.8	89.6	84.8

* Restricted below diagonal and unrestricted above diagonal.

Recombinant plasmids from the Octopus Spring-II library were derived from RNA obtained from enzymatic lysis of the top 0–5 mm vertical zone of biological activity within the 55 °C mat¹³. RNA for the Octopus Spring-III library was obtained from French Pressure Cell lysis (twice at 20,000 psi) of the top 0–1 mm vertical zone of oxygenic photosynthesis of the 55 °C mat¹³. 16S rcDNA synthesis and construction of recombinant libraries were as described⁹. Sequences were determined using Sequenase (US Biochemical), primers complementary to regions of the cloning vector sequence adjacent to the cloning site or to universally conserved segments of 16S rRNA sequence, and sequencing gels and conditions which have been previously detailed¹⁸. (The sequence data in this publication have been submitted to the EMBL database under the accession numbers X52544 to X52551.) Sequences were aligned and similarities between sequence pairs were determined^{16,17}. Sequences ranged from 118 to 588 nucleotides in length, always starting adjacent to the priming site (nucleotide 1,392 in the *E. coli* 16S rRNA sequence). Restricted analysis (below diagonal) excludes variable regions corresponding to positions 826–874, 991–1,045, 1,115–1,182, and 1,249–1,286 of the *E. coli* sequence, as well as gaps found in only one out of a set of sequences. Unrestricted analysis (above diagonal) includes all positions.

Similarity values among 16S rcDNA sequences retrieved from the Octopus Spring mat (Table 1) reveal eight distinct sequence types (A–H) which must be contributed by eight different community members. Five sequences are identical in both restricted and unrestricted analysis (type-A). Another sequence (type-B) is highly related, but eleven differences distinguish it from type-A sequences. Two other sequence types (C and D) are revealed as pairs which are also identical in restricted and unrestricted analysis. Four additional sequence types (E, F, G and H) are represented only once.

None of the eight Octopus Spring 16S rcDNA sequence types is identical with a 16S rRNA sequence of any microorganism isolated from the mat or other similar geothermal habitat (Table 2). The highest similarity is between type-C sequences and the sequence of *C. aurantiacus* (94.7% in restricted analysis), but the lack of identity is verified by a lower similarity value

(85.9%) in unrestricted analysis. Consequently, we believe that the eight sequence types we have recovered from the mat are contributed by microbial inhabitants which have not been cultured from this community. Our confidence in this interpretation comes from several lines of reasoning. First, the redundancy of type-A, type-C and type-D 16S rcDNA sequences (Table 1) and the reproducibility of 16S rRNA sequence determination^{18–20} demonstrate the high fidelity of these methods in copying 16S rRNA sequence information. Second, the method is equally faithful in recovering 16S rcDNA sequences of isolated bacteria grown in pure culture²⁰. Third, the 16S rcDNA sequence data provide convincing evidence, at both the primary and secondary structure level, that similarity values are based on unambiguous nucleotide differences occurring at nonrandom positions in the molecule (mainly in variable regions as expected, and with compensatory differences at complementary positions in

TABLE 2 Sequence similarity between Octopus Spring cyanobacterial mat 16S rcDNAs and 16S rRNAs of pure cultured mat inhabitants

Pure culture	Percent restricted similarity with 16S rcDNA sequence type							
	A*	B	C*	D*	E	F	G	H
<i>Synechococcus lividus</i>	90.6	91.7	77.2	87.3	80.1	83.5	77.3	83.4
<i>Chloroflexus aurantiacus</i>	86.2	87.7	94.7	75.2	85.8	80.0	81.9	83.2
<i>Thermus aquaticus</i>	80.2	80.4	80.9	84.0	83.4	88.0	83.8	86.6
<i>Thermomicrobium roseum</i> †	87.2	87.3	85.0	73.6	82.6	73.6	81.7	82.5
<i>Herpetosiphon aurantiacus</i> †	85.1	86.3	89.7	73.9	86.5	77.7	84.1	80.4
<i>Isophaera pallida</i>	85.4	84.9	83.8	77.8	82.2	78.8	80.3	83.0
<i>Thermobacteroides acetothylis</i>	89.1	89.3	81.0	83.8	86.0	85.9	88.6	88.6
<i>Thermoanaerobacter ethanolicus</i>	86.8	87.0	77.0	83.8	83.0	85.9	87.0	86.8
<i>Thermoanaerobium brockii</i>	86.7	87.0	76.9	83.8	83.0	85.9	86.5	86.7
<i>Clostridium thermohydrosulphuricum</i>	89.1	89.3	80.9	84.4	86.4	87.1	87.5	89.1
<i>Clostridium thermoautotrophicum</i>	91.2	91.9	84.0	86.5	87.6	89.2	89.4	89.9
<i>Clostridium thermosulfurogenes</i>	87.8	88.7	80.8	82.0	82.0	83.0	81.4	85.4
<i>Thermodesulfobacterium commune</i>	82.0	83.1	80.2	84.3	86.7	88.0	83.0	85.2
<i>Methanobacterium thermoautotrophicum</i>	75.8	77.1	75.4	65.9	71.1	70.7	73.6	75.1

The pure-cultured microorganisms were isolated from, or are thought to inhabit, the Octopus Spring mat community. Sequences of pure cultures have been previously published^{18–20,28}. Data are from restricted analysis. Similarity percentages are not absolute because the number of nucleotides compared varies as a function of the length of overlap between isolate and plasmid sequences, ranging from 83 to 381 bases with an average 170 bases. For comparisons in this range phylogenetic interpretations of similarity percentages can be based on interkingdom, interphylum and intraphylum similarities of 67.1–82.1%, 76.4–88.4% and 85.9–100%, respectively, as determined from the pure culture data set analysed under the same constraints.

* Consensus sequences determined from replicated sequences of this type.

† Not isolated from or observed in the Octopus Spring cyanobacterial mat.

double-stranded regions, see Fig. 1). Finally, we have made similar observations in other Octopus Spring 16S rDNA libraries.

One valuable application of the use of 16S rRNA sequences in characterizing microbial communities is to provide insight into the phylogenetic types of uncultured community members, as well as a basis for designing hybridization probes to study their autecology^{10,11,21-23}. Our data show that all eight mat sequence types are of eubacterial rather than archaeobacterial origin, as similarity values with eubacterial isolates are typical of inter- or intraphylum difference, whereas similarity values with *Methanobacterium thermoautotrophicum*, the only archaeobacterial isolate from the mat, are typical of interkingdom difference. Two sequence types can be placed within specific eubacterial phyla. The 94.7% similarity between type-C and *C. aurantiacus* sequences suggests that these sequences are contributed by a member of the green non-sulphur eubacterial phylum. The type-H sequence, while dissimilar to sequences of mat isolates, exhibits 94.3% similarity with the sequence for *Spirochaeta halophila*, suggesting it is contributed by an as yet uncultivated spirochaete inhabitant of the mat. These affinities are further supported by preliminary distance matrix tree analysis and by nucleotide and oligonucleotide signatures characteristic of members of these phyla^{8,24}. Type-A and -B sequences show 90.6% to 91.9% similarity with sequences representative of the cyanobacterial (*S. lividus*) or Gram-positive (*Clostridium thermoautotrophicum*) eubacterial phyla. Whereas nucleotide and oligonucleotide signatures do not resolve placement into one of these phyla, the sequences show greater similarity with that of another cyanobacterium, *Anacystis nidulans*²⁵ (93.2% and 94.5% in restricted analysis for type-A and -B sequences, respectively). We will explore further the possibility of a cyanobacterial origin of these sequences, as this would challenge the notion derived from microscopic and enrichment culture evidence that *S. lividus* is the sole cyanobacterium building the mat. Other sequence types bear too little similarity with known eubacterial phyla to presently enable further assignment; some of these may represent novel phyla.

The ecological message is clear—this well-studied microbial mat contains a large number of community members which have never been cultured from this habitat. The same is almost certainly true of most, if not all, microbial communities, as our knowledge of them is similarly based on culture-dependent methods. Analysis of marine microflora by 16S rRNA-based methods has led to similar observations²⁶. Further work will be required before we can know more precisely the percentage of extant microorganisms pure culture collections represent. In this community the number of new inhabitants so far suggested by our method is comparable to the number of cultured inhabitants whose 16S rRNA sequences have not yet been detected. From this we infer that the percentage of uncultured inhabitants is likely to be, as speculated, quite high. This new culture-independent method is beginning to reveal some of the unexplored diversity within the microbial world and should eventually provide a more objective approach by which to understand the composition of natural microbial communities²⁷. □

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Rapid kinetics of second messenger formation in olfactory transduction

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OLFACTORY transduction is thought to be mediated by a membrane-bound receptor protein initiating a multistep reaction cascade which ultimately leads to a depolarizing generator current^{1,2}. There is considerable evidence for the involvement of adenylate cyclase in vertebrate olfactory transduction^{3–6}, and some data indicate that phospholipase C may have a central role in insect olfaction⁷. However, one must show that odorants not only stimulate enzyme activity but also induce changes in concentrations of relevant second messengers. One important criterion for a candidate second messenger of chemo-electrical transduction is that its formation must precede the onset of the odorant-induced membrane permeability changes which proceed on a subsecond time-scale⁸. Here we report an odorant-induced, transient accumulation of cyclic AMP in isolated olfactory cilia from rats, and the generation of inositol trisphosphate in antennal preparations from insects, both of which show subsecond time courses that are sufficiently rapid to mediate the odorant-regulated permeability of olfactory receptor cells.

Figure 1 shows the odorant-induced changes in second messenger concentrations. In a preparation of isolated olfactory cilia from rat, the basal level of 78 pmol cAMP per mg protein was close to the detection limit. Upon application of 1 µM isomenthone (Fig. 1a), the concentration of cAMP rapidly increased, reaching a maximal level of 303 pmol per mg protein between 25–50 ms and declined thereafter to the basal level in 250–500 ms. In contrast, the concentration of inositol 1,4,5-trisphosphate (IP₃) was not affected. In antennal preparations from cockroach, the application of 60 pM periplanone B, a specific sex pheromone⁹, induced the formation of IP₃ with a similar time course. From a basal level of 65 pmol per mg protein, the peak concentration (2,540 pmol mg⁻¹) was reached 50 ms after application of the pheromone (Fig. 1b). The raised IP₃ level rapidly decayed, returning to the original concentration within 100 ms. In antennal preparations changes in the levels of cAMP were never observed.

Both second messenger transients were dependent on odorant

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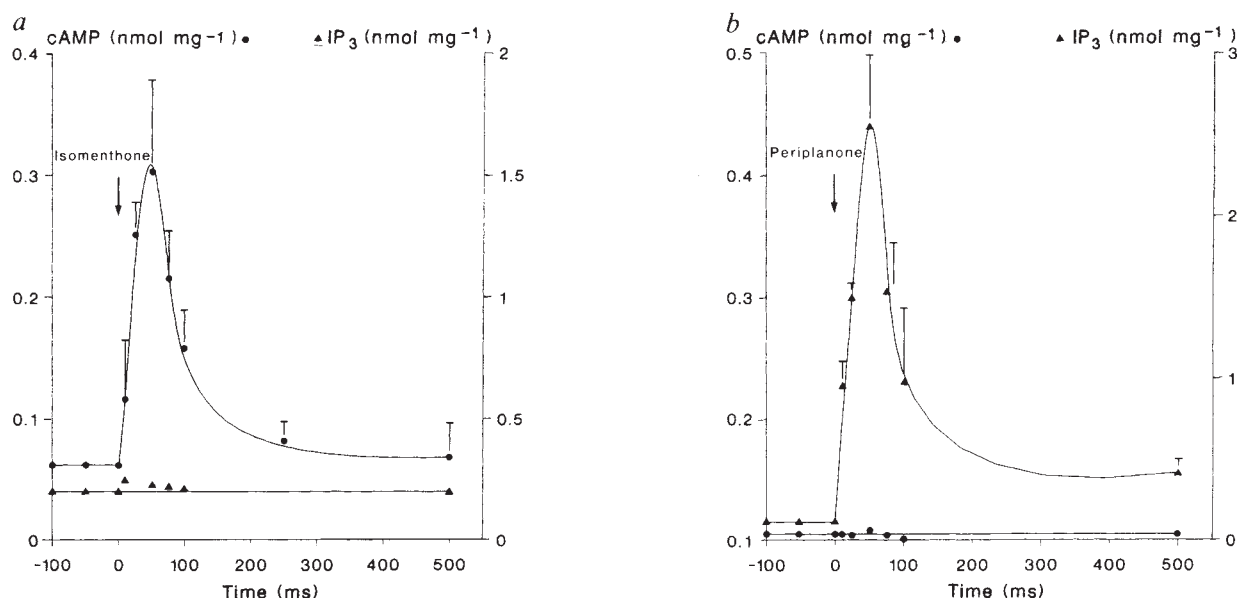


FIG. 1 Rapid kinetics of odorant-induced accumulation of second messengers in isolated cilia from the olfactory epithelium of rats (a) and antennal homogenates from cockroaches (*Periplaneta americana*) (b).

METHODS. Cilia were prepared from Sprague-Dawley rats^{3,16}. Isomenthone was prepared as a stock solution in dimethyl sulphoxide (DMSO) and diluted in reaction buffer. The concentration of DMSO never exceeded 0.1%, a concentration that did not interfere with the assay. Antennae from male or female cockroaches were homogenized in 50 mM MOPS, 200 mM NaCl, 10 mM EGTA, 2.5 mM MgCl₂, 1 mM DTT (pH 6.9). The homogenate was supplemented with 0.05% (w/v) sodium cholate, 1 mM ATP, 1 μ M GTP and appropriate amounts of CaCl₂ to provide a free Ca²⁺ concentration of 0.01 μ M. Periplanone B stored in hexane was dissolved in DMSO to give a stock solution of 12 μ M; appropriate amounts were added to a solution containing the same ingredients as the homogenate medium. A rapid quench device with three syringes was used to measure the subsecond kinetics of odorant-induced changes in cAMP and IP₃ levels. The first syringe contained

the tissue preparation, the second syringe was filled with solution containing the odorant at appropriate concentrations and the third syringe contained 10% perchloric acid. The device could mix and quench samples at time intervals ranging from 8 ms to 0.5 s. Activation of the different syringes was controlled by an IBM AT computer. The quenched preparations were immediately placed on ice and vortexed. The samples were spun for 5 min at 2,500g at 4 °C. The supernatants were removed and neutralized by the addition of 1,1,2-trichlorotrifluoroethane (Freon)/tri-*n*-octylamin (1:1, v/v), vortexed and spun for 2 min at 300g. The neutralized supernatant was used for radioimmunoassays. The determination of cAMP (●) followed Steiner *et al.*¹⁷. IP₃ (▲) was estimated according to Palmer *et al.*¹⁸. Protein content was measured¹⁹ with BSA as a standard. It has been reported that in soluble systems acid or alkali quenching requires ≤ 1 –3 ms (ref. 20). The time axis in Figs 1 and 2 represents the time at which acid ejection was initiated. Data shown are the means of 4–6 experiments $\pm$ s.d.

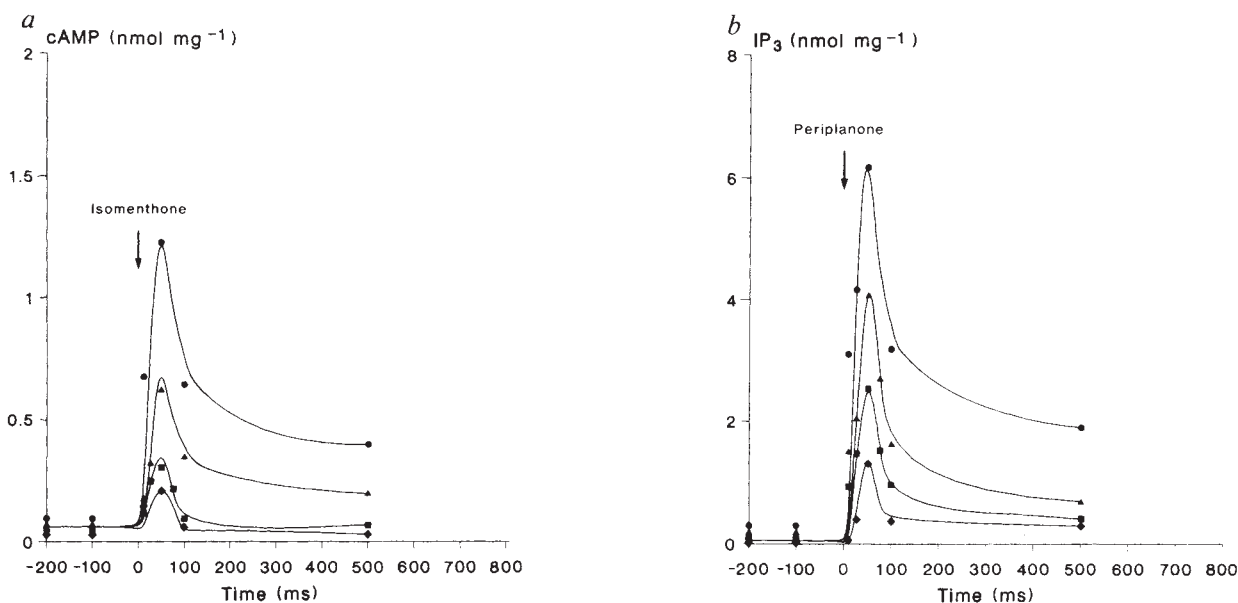


FIG. 2 Onset and decay of a rise in second messenger concentration on exposure to various odorant doses. a, Time course of cAMP accumulation in rat cilia induced by application of 10^{-7} – 10^{-4} M isomenthone. The concentration of cAMP is clearly odorant dose-dependent; the decay time is significantly prolonged in the presence of higher odorant concentrations. Isomenthone concentration: ●, 100 μ M; ▲, 10 μ M; ■, 1 μ M; ◆, 0.1 μ M.

b, Changes in the level of IP₃ in antennal preparation after application of 6×10^{-12} – 6×10^{-9} M periplanone B. A concentration-dependent accumulation in the 50 ms range is seen; at higher pheromone concentration a raised level of IP₃ can be seen even after 500 ms. Data are the means of 4–6 experiments. Periplanone concentration: ●, 6 nM; ▲, 600 pM; ■, 60 pM; ◆, 6 pM.

concentration over several orders of magnitude (10^{-7} – 10^{-4} M isomenthone in rat cilia (Fig. 2*a*); 6×10^{-12} – 6×10^{-8} M periplanone B in cockroach antennae (Fig. 2*b*). In addition, the time course of recovery to the baseline levels of cAMP and IP_3 was odorant dose-dependent. Whereas the levels decayed very rapidly at low odorant concentrations, raised levels of cAMP (in rat cilia) and IP_3 (in insect antennal preparations) were observed for many seconds after the application of high concentrations of odorants. This discrepancy is probably due to the time required to inactivate higher odorant concentrations, and may explain why stimulatory effects of the odorants on either adenylate cyclase^{3,4} or phospholipase C (ref. 7) were only seen at rather high concentrations of odorant.

The stimulatory effects on second messengers were quite

specific, as odorants did not induce formation of second messengers in non-sensory excitable tissue such as nerve and muscle, and the pheromone effect was only observed on preparations from male antennae.

In both systems the rapid odorant-activated second messenger cascade seems to be GTP-dependent. Neither GTP nor odorants when tested alone showed any significant effect on cAMP or IP_3 levels (Fig. 3). The low level of odorant stimulation in the absence of added GTP may reflect endogenous stores of GTP. But application of odorant together with GTP synergistically induced an accumulation of cAMP in rat cilia, and IP_3 in the antennal preparation (Fig. 3*a, c*).

The view that the dynamic regulation of second messenger formation by odorants requires GTP hydrolysis and the involve-

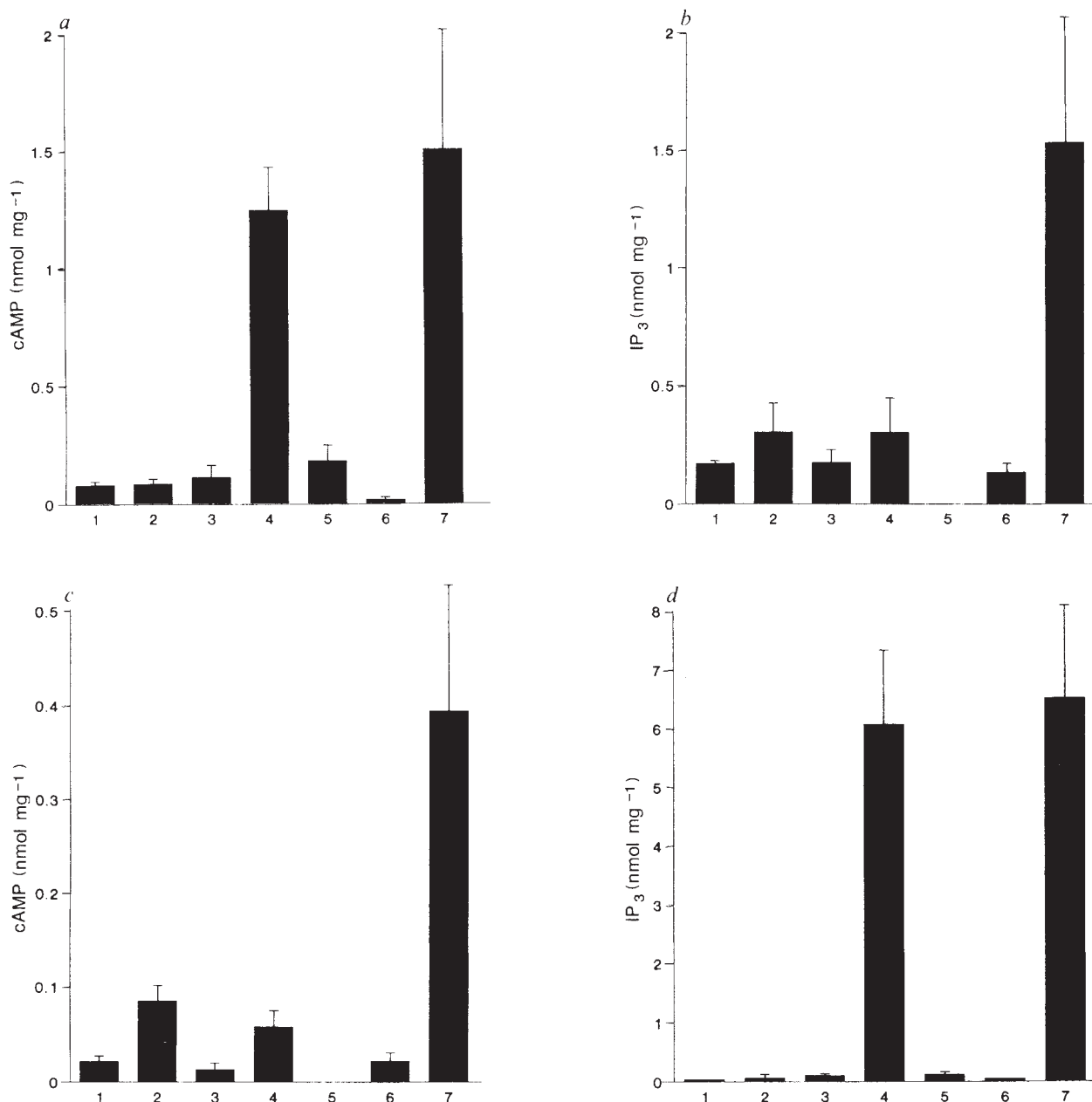


FIG. 3 Effects of guanine nucleotides on the levels of cyclic cAMP and IP_3 in rat olfactory cilia and cockroach antennae. *a*, Concentration of cAMP in isolated cilia preparation from rat olfactory epithelia; *b*, IP_3 concentration in rat olfactory cilia; *c*, concentration of cAMP in cockroach antennal homogenate; *d*, IP_3 concentration in the antennal preparation. Cilia or antennal preparation were supplemented with various agents and incubated for 50 ms. Concentrations of cyclic AMP and IP_3 were determined as described

in Fig. 1. 1, Control; 2, +1 μ M GTP; 3, +100 μ M isomenthone (*a, b*), +0.6 nM periplanone B (*c, d*); 4, +1 μ M GTP plus 100 μ M isomenthone (*a, b*), +1 μ M GTP plus 0.6 nM periplanone B (*c, d*); 5, +10 μ M GDP- β -S plus 100 μ M isomenthone plus 1 μ M GTP (*a, b*), +10 μ M GDP- β -S plus 0.6 nM periplanone B plus 1 μ M GTP (*c, d*); 6, +10 μ M GDP- β -S; 7, +10 μ M GTP- γ -S. Data are the mean of 4–6 experiments $\pm$ s.d.

ment of G proteins is further supported by experiments using guanosine nucleotide derivatives. Whereas the basal levels of cyclic AMP and IP_3 were hardly affected by GDP- β -S, this analogue, which interferes with the activation of G proteins¹⁰, almost completely prevented the odorant-activated formation of cyclic AMP in rat cilia, as well as the generation of IP_3 in antennal preparations (Fig. 3a, c). In contrast, GTP- γ -S, the slowly hydrolysing analogue of GTP (ref. 10), induced rapid formation of cAMP in cilia and IP_3 in antennal preparations even in the absence of odorants. The concentrations determined 50 ms after GTP- γ -S application exceeded the levels induced by the highest concentrations of odorants (Fig. 3). Because this GTP derivative hydrolyses slowly, the decay time of both metabolites was considerably prolonged in the presence of GTP- γ -S. For example, highly elevated transmitter concentrations were detectable even after 500 ms. GTP- γ -S not only induced the generation of cAMP, but also of IP_3 in rat cilia, as well as in insect antennae (Fig. 3b, d). This observation clearly indicates that the two signalling pathways are present in both sensory tissues, but that only one system is selectively activated by the respective odorant, probably through specific receptors and G proteins. Although specific odorant receptors have not yet been identified, an olfactory-specific G protein α -subunit, termed G_{olf} , which shares extensive amino-acid identity with the stimulatory G protein G_{sa} , has recently been found in rats¹¹.

This study provides evidence for the notion that different second messengers may be involved in olfactory transduction in vertebrates and arthropods. Also, we have established that the subsecond time course of odorant-induced increases in second messenger concentration (cAMP in rat olfactory cilia and IP_3 in insect antennae) precede the odorant-induced activation of a depolarizing generator current, which appears only after a latency of several hundred milliseconds^{8,12}. The discovery of such rapid increases in concentration of second messenger is of particular significance as they represent the only known chemical changes in olfactory tissue preceding the electrical response to odorants. All previous biochemical studies concerning olfactory transduction have relied on enzyme assays performed in the minute or second time-range. Hitherto, specific changes in concentration of prospective intracellular messenger molecules have not been demonstrated in response to odorants^{3,4}. To ascertain the role(s) of cAMP in vertebrate and IP_3 in insect olfactory transduction it will be necessary to determine the site(s) of action of these prospective second messengers on components of the transducing reaction cascade. In this respect, the recent observation of Nakamura and Gold⁵, that excised patches from the ciliary membranes of isolated olfactory sensory neurons contain a cAMP-gated cation channel, is of particular relevance. Olfaction thus seems to bear a striking molecular similarity to retinal photodetection in which light-induced, G-protein mediated changes in the concentration of cGMP occur on a subsecond timescale^{13,14}, cGMP then triggering the activation of cation channels¹⁵. □

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Virus-induced autoantibody response to a transgenic viral antigen

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THE induction of autoantibodies and their possible role in the pathogenesis of autoimmune disease are poorly understood. Involvement of infectious agents has been suspected, but direct evidence is sparse^{1–4}. Whether immunological unresponsiveness to self by antibody-forming B cells is maintained by clonal abortion⁵, clonal anergy^{6–8} or suppression⁹, or how the scenario of interactions between helper T cells, B cells and antigen-presenting cells^{10,11–13} is distorted in autoantibody responses, is being analysed and widely debated^{3,6–8,14–17}. To evaluate tolerance of neutralizing B-cell responses^{12,18} we used transgenic mice expressing the cell membrane associated glycoprotein (G) of vesicular stomatitis virus (VSV) as self-antigen. We show that autoantibodies to VSV-G cannot be induced by VSV-G in adjuvant or by recombinant vaccinia virus expressing VSV-G, but are triggered by infection with wild-type VSV. The data show that helper T-cell tolerance is crucial in maintenance of B-cell non-reactivity^{2,16,19,20} and that cognate T–B recognition is necessary to break tolerance of self-reactive B cells. These results may help to understand mechanisms of virus-induced autoimmunity.

The experiments were based on the following experimental features offered by VSV and by VSV-G transgenic mice.

(1) IgM B-cell responses to VSV-G are triggered in the strict absence of T help; the IgG response, on the other hand, is strictly T-help dependent¹⁸. (2) VSV-Indiana wild-type virus (VSV-IND wt) and VSV-New Jersey wild-type (VSV-NJ wt) induce cross-reactive virus-specific cytotoxic T cells^{21,22} and cross-reactive helper T cells, when assayed for T help in dinitrophenyl-specific antibody responses¹⁸. (3) The neutralizing antibodies induced by VSV-IND and VSV-NJ are directed only against VSV-G and are strictly not cross-reactive^{23,24}. The VSV-G neutralizing determinant may here be regarded as a haptenic B-cell determinant of biological relevance. (4) Transgenic mice expressing VSV-G IND were generated as shown in Fig. 1. Development in these mice of autoantibody–VSV-IND neutralizing antibody—to the VSV-G IND self-antigen could be monitored very sensitively. (5) Two distinct pairs of viruses were available: VSV-IND wt and VSV-NJ wt which contain the respective G proteins in the viral envelopes, and recombinant vaccinia viruses containing VSV-G IND (vacc-G IND) or VSV-G NJ (vacc-G NJ)²². In contrast to VSV wt, the recombinant vaccinia viruses do not contain detectable VSV-G in their envelope (B. Moss, personal communication).

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TABLE 1 Primary neutralizing anti-VSV-G responses in VSV-G IND transgenic mice

Mouse line or strain	Titres of neutralizing activity in serum ($\log_2 \times 10^{-1}$) on the indicated day after i.v. infection with 2×10^6 PFU of vacc-G IND (experiment 1)						vacc-G NJ (experiment 2)	
	day 4		day 10		day 10		day 10	
	anti-VSV-IND IgM + IgG	IgG	anti-VSV-IND IgM + IgG	IgG	anti-VSV-IND IgM + IgG	IgG	anti-VSV-NJ IgG	IgG
163	3	<1	<1	<1			7	
	3	<1	<1	<1			7	
	2	<1	<1	<1			6	
207	<1	<1	<1	<1			5	
	<1	<1	<1	<1			6	
	<1	<1	<1	<1			7	
29	4	<1	3	3			5	
	4	<1	7	6			5	
	5	<1	6	5			6	
C57BL/6	8	<1	9	8			5	
	7	<1	10	9			6	
	7	<1	9	8			5	
C3H/HeJ	6	<1	8	7			6	
	6	<1	7	7			6	
	5	<1	8	6			7	

Infection with a mixture of 2×10^6 PFU vacc-G IND + 2×10^6 PFU vacc-G NJ (experiment 3)						
anti-VSV IND			anti-VSV NJ			
day 4	day 7	day 12	day 4	day 7	day 12	day 12
163	2	<1	<1	2	7	7
207	<1	<1	<1	3	8	8
C57BL/6	3	5	4	1	7	5
C3H/HeJ	2	2	6	4	6	7

The recombinant vaccinia viruses²² were a gift of Dr B. Moss, NIH. Neutralizing serum titres were determined in individual sera against both VSV-IND and VSV-NJ as described elsewhere¹⁸; only titres against the homologous VSV are shown as titres against the heterologous VSV were all <1. Titres represent twofold dilution steps of sera starting with 1:40. IgM+IgG, titre of untreated sera representing IgM on day 4, and mostly IgG plus some IgM on day 7 and later in normal control mice¹⁸. IgG represents titres of sera treated with 0.1 M β 2-mercaptoethanol. Values shown are individual titres (experiments 1 and 2) or means of titres of three mice (experiment 3); s.e.m. values were $\leq \pm 1$ titration step.

Transgenic mice expressing VSV-G IND under the control of various promoters were generated (Fig. 1). VSV-G IND could not be detected by a sandwich enzyme-linked immunoabsorbent assay (ELISA) at concentrations less than 60 ng per ml of serum, perhaps because binding of VSV-G to serum lipoproteins and natural antibodies limits the detection level²⁵; no correlation between tolerance and concentration of serum VSV-G IND could therefore be established^{8,16,26}. However, VSV-G IND messenger RNA was readily detected in various organs depending on the promoter used (Fig. 1). Mice of strain 163 infected with vacc-G IND generated a small transient IgM response on day 4, whereas mice of strain 207 did not (Table 1). By contrast, mice of line 29, not expressing G, made a normal IgM and IgG response; therefore this line was not studied further. None of the transgenic mice developed measurable IgG titres on days 10 and 20.

Even after boosting with 5×10^7 plaque-forming units (PFU) vacc-G IND intraperitoneally (i.p.) 20 days after the primary infection, only a small transient IgM response, but no IgG response, was found in line 163 and none in line 207 (Table 2, experiment 1). Because mice were derived from (C57BL/6 $\times$ C3H) F2 mice, C57BL/6, C3H or negative littermates were used as controls; they made a normal IgM (day 4) and IgG (day 10) response to the same vacc-G IND virus (Table 1, experiment 1). Therefore transgenic mice were tolerant to VSV-IND G except for the transient IgM response in line 163.

Tolerance was specific, as all transgenic mice analysed showed a neutralizing anti-VSV NJ response similar to control mice after infection with the vacc-G NJ recombinant virus (Table 1, experiment 2). Analogous results were obtained in mice co-infected simultaneously with vacc-G IND and vacc-G NJ (Table

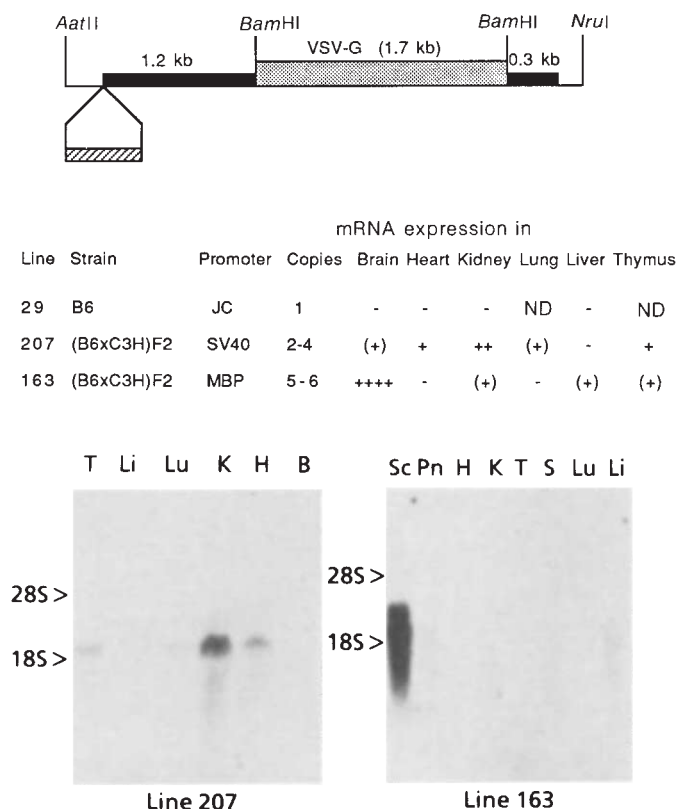


FIG. 1 Characterization of VSV-G transgenic mice. *a*, DNA fragments used for microinjection of mouse embryos; *b*, summary of mRNA expression in different organs as derived from northern-blot analysis; *c*, examples of northern blots obtained with mRNA from different tissues of line 207 and 163. T, thymus; Li, liver; Lu, lung; K, kidney; H, heart; B, brain; Sc, spinal cord; Pn, peripheral nerve; S, spleen. Positions of ribosomal RNA size markers are shown. ND, not determined.

METHODS. The injected fragments contained (5' to 3'): 57 base pairs (bp) of vector sequence (pBr322) (thin line) bounded by the *AatII* site; either one of three inserts (hatched); 1.2 kilobase pairs (kbp) of the SV40 late splice region (solid box); the entire coding regions for the VSV-G protein (serotype Indiana³⁸) (stippled box); 0.3 kbp of the 3' SV40 late region including its poly(A) addition signal (solid box); 78 bp of vector sequence up to the *NruI* site. The promoter inserts were: the 286-bp *PvuII*-*HindIII* fragment of the early promoter of the human papovirus JC (line 29); the 342-bp *PvuII*-*HindIII* fragment of the early promoter of JC (line 207)⁴⁰; an *EcoRI*-*BamHI* fragment that contains 6.5 kb of mouse genomic sequence found upstream of the myelin basic protein (MBP) transcription initiation site and the first three nucleotides of the first MBP exon, followed by a synthetic *Sall*-*BamHI* linker (line 163). To obtain this sequence, a 6.5-kb *EcoRI*-*XbaI* fragment that extends to position -54 of the MBP transcription initiation site was subcloned from λ MBP1, a 20-kb clone isolated from a genomic BALB/c EMBL3 library. Insertion of an oligonucleotide linker at the *XbaI* site recreates the normal mouse MBP initiation site through position +3 and adds *Sall* and *BamHI* restriction sites. The *AatII*-*NruI* fragments isolated and purified from the respective plasmids were injected at $2 \text{ ng } \mu\text{l}^{-1}$ into pronuclei of fertilized C57BL/6 or (C57BL/6 $\times$ C3H) F2 embryos according to standard procedures. Transgenic founders and their offspring obtained by brother-sister matings or backcrosses of positive males to C57BL/6 females were identified by Southern-blot analysis of tail DNA. For mRNA analysis, 10 μg total RNA isolated from the respective organs of 4-7-week-old mice was separated by agarose gel electrophoresis, blotted onto nitrocellulose membranes, and hybridized with radiolabelled VSV-G cDNA. For peripheral nerve, the RNA of two sciatic nerves was applied. The relative amounts of mRNA (indicated by the number of plus signs in *b*) are based on the intensities obtained from the autoradiograms of the northern blots.

TABLE 2 Primary and secondary neutralizing anti-VSV-G IND responses in VSV-G IND transgenic mice

Mouse line or strain	Titres of neutralizing activity in sera ($\log_2 \times 10^{-1}$) on the indicated day								
	day 4		day 10		day 20	day 4		day 10	day 20
	IgM + IgG	IgG	IgM + IgG	IgG	IgG	IgM + IgG	IgG	IgG	IgG
Experiment 1									
	Primary injection with 5×10^7 PFU vacc-G IND					Secondary injection with 5×10^7 PFU vacc-G IND			
163	3-5	<1	<1	<1	<1	1-2	<1	<1	
207	<1	<1	<1	<1	<1	<1	<1	<1	
C57BL/6	5-8	<1	6-7	5-6	8-9	8-9	8-9	7-8	
Experiment 2									
	Primary injection with 5 μ g G IND CFA					Secondary injection with 5 μ g G IND			
207	<1	<1	<1	<1	<1	<1	<1	<1	<1
163	<1	<1	<1	<1	<1	<1	<1	<1	<1
C57BL/6	<1	<1	<1	<1	3	5	5	9	10
C3H	<1	<1	<1	<1	5	9	8	10	10
	Primary injection with 5 μ g G NJ IFA					Secondary injection with 5 μ g G NJ			
207	<1	<1	<1	<1	4-5	9	8	10	11
163	<1	<1	<1	<1	2-3	10	9	11	11
Experiment 3									
	Primary injection with 2×10^6 PFU VSV-IND wt					Secondary injection with 10^8 PFU VSV-IND wt			
163	5-6	<1	6-7	1-2	5-6	10-11	10-11	>12	>12
207	<1-1	<1	6-7	1-2	5-7	9-10	8-10	9-10	10-11
C57BL/6	7-8	<1	9-10	8-9	8-9	11-12	10-11	10-12	10-11
Experiment 4									
	Primary injection with 2×10^4 PFU VSV-IND wt								
207	<1-1	<1	2-5	<1-2	5-6				
C57BL/6	5-6	<1	9-11	8-9	8-9				
Experiment 5									
	Primary injection with 2×10^6 PFU vacc-G IND					Secondary injection with 10^8 PFU VSV-IND wt			
163	2-3	<1	<1	<1	<1	4-5	<1	2-3	5
207	<1	<1	<1	<1	<1	3-4	1-2	3-4	4-6
CB57BL/6	7-8	<1	8-9	7-8	8-9	10-12	10-12	9-12	8-12

For infection of mice with vacc-G IND and standard methods, see legend to Table 1. Mice were reinjected with VSV-G IND wt virus or vacc-G IND on day 20. In experiment 2, mice were injected i.p. with 5 μ g purified VSV-G as described in ref. 18 in CFA or IFA. These mice were boosted with 5 μ g purified VSV-G i.v. on day 20. In experiments 3-5, mice were infected i.v. with VSV-IND wt, grown on BHK cells. Only titres against the homologous VSV are shown; no titres were found against the heterologous VSV-NJ. Also, sera taken before infection did not show detectable titres against VSV-IND. Values are ranges of three to four individual serum titres.

1, experiment 3). These data also show that tolerance to VSV-G IND does not impair help against VSV-G NJ. This is readily explained by the known differences between the VSV-G IND and VSV-G NJ amino-acid sequences of about 50% (refs 27, 28).

To study tolerance further, transgenic mice and control mice were immunized with purified G IND in complete Freund's adjuvant (CFA) and boosted with purified VSV-G IND: no IgM or IgG was found in transgenic mice. By contrast, control mice mounted a slow primary response but an immediate and normal secondary response. Both strains of transgenic mice responded to purified G NJ in incomplete Freund's adjuvant (IFA) (Table 2, experiment 2). Therefore, tolerance in transgenic mice was VSV-G IND specific; increasing the concentration of self VSV-G IND in lymphoid organs alone or together with independent stimulation of anti-VSV-G NJ (Table 1, experiment 3) or anti-vaccinia T help was not sufficient to break tolerance.

To evaluate the primary antibody response to VSV-IND wt transgenic lines 163 and 207 were infected intravenously (i.v.) with 2×10^6 PFU VSV-IND wt. On day 4 the neutralizing titre of the IgM response of line 163 mice reached titres of 5 (1:640) compared with titres of 7 (1:2,560) in control C57BL/6 mice (Table 2, experiment 3). Line 207 mice showed a measurable IgM response but only a marginal IgG response by day 10, when control C57BL/6 had switched to the usual high IgG titre. Both 163 and 207 transgenic mice had switched to IgG by day 20. Thus the VSV-G IND transgenic mice could be induced to mount a neutralizing anti-VSV-G IND IgM and IgG antibody response with VSV-IND wt virus as immunogen (even at low doses, 2×10^4 PFU, Table 2, experiment 4) but, surprisingly, not with vacc-G IND.

Break of tolerance could not easily be explained by quantitative differences because neither virus replicates extensively in extraneuronal tissue when injected peripherally into adult mice^{29,30}. The results of our experiments on tolerance breakdown also fit the results observed after secondary infections (right half of Table 2). Whereas transgenic mice primed with VSV-IND wt mounted a secondary response with rapid increase of IgG titres by day 4 (Table 2, experiment 3), mice primed with vacc-G IND made a primary response to VSV-IND wt (Table 2, experiment 5, low IgM on day 4, slow switch to IgG by day 20). The latter result reveals that the earlier B-cell encounter with antigen (priming with vacc-G IND) in the absence of T help does not result in B-cell anergy in this experiment (see Table 2, experiment 3), as suggested by other experiments⁸. Thus transgenic mice were neither able to respond to IND-G in CFA nor to infection with vacc-G IND virus; surprisingly, infection with VSV-IND wt, however, could break their B-cell (IgG) unresponsiveness.

Many viruses (for example, herpes virus, Epstein-Barr virus, coxsackie and retroviruses^{1,3,31}), particularly if persistent, have been suspected to be involved in triggering autoantibodies and autoimmune disease. Infectious agents may code for both strong T-helper determinants and antigen fragments that resemble normal self-antigens recognizable by autoantibodies (molecular mimicry)³. The implied mechanism was that T help to viral peptide antigens on cells could trigger, by means of released lymphokines, cross-reactive B cells recognizing self-antigens on the same cell.

Our results with vacc-G IND indicate that this view may have to be modified. First, unresponsiveness at the B-cell level (depen-

dent on antigen concentration^{16,26}) may be incomplete or absent compared with the solid tolerance at the helper T-cell level under a given expression level of a 'self'-antigen (so-called split tolerance)². But provided that T help is absent, B-cell unresponsiveness to self is generally maintained, except for situations in which B cells may be transiently activated directly by specific (as here for VSV-inducing IgM) or nonspecific polyclonal stimulation³². Second, the data indicate that, in contrast to the mechanism mentioned above, T help has to be guided directly to the specific B cell to trigger self-reactive B cells; that is, interleukins released by vaccinia-specific helper T cells alone without direct specific contact between T and B cell are insufficient to break B-cell unresponsiveness^{20,33}.

Thus, at some crucial and limiting step, autoreactive B cells themselves must specifically take up self-antigen (VSV-G IND) and present 'new' T helper determinants in order to become induced. Accordingly we interpret the difference between VSV-IND wt and VSV-G IND in CFA or vacc-G IND to reflect specific uptake of VSV-IND wt, which expresses G on the viral envelope, by neutralizing B cells specific for IND. After processing the complete virus particle, these B cells may receive T help specific for (MHC) class II associated, for example, with nucleoprotein (NP) determinants (to which they are not tolerant)³⁴. In VSV-G transgenic mice, relevant T helper determinants can derive neither from G IND because of tolerance, nor from vaccinia peptides because vacc-G IND expresses no G IND on the virus envelope, and therefore cannot be taken up preferentially and processed by neutralizing antibody-producing B cells specific for VSV-IND.

An alternative explanation for the apparent lack of T help induced by vacc-G IND in transgenic mice may be that by infection with VSV-IND wt, T help may be triggered to (non-tolerized) 'novel' G peptides that are differently processed in specific B cells from the normal self-G peptides generated in transgenic cells expressing G or in cells infected with vacc-G IND (as might be extrapolated from other evidence³⁴⁻³⁷).

In conclusion, the results document a crucial need for T help against nonself to break tolerance of B cells to self, and indicate that IgG autoantibody responses may be triggered only after both self and foreign antigens have been taken up, processed, and presented by self-reactive B cells. The model presented may therefore reveal generalized mechanisms by which viruses trigger (and if persistent, may help to maintain) IgG autoantibodies against cell-surface self-antigens potentially involved in autoimmune diseases. □

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Association of CD2 and CD45 on human T lymphocytes

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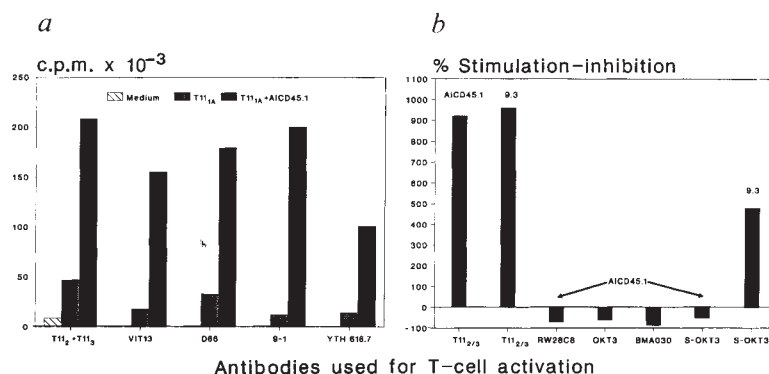
At least two membrane receptors have been defined through which human T lymphocytes can be induced to proliferate and differentiate, namely the CD3-Ti antigen receptor complex¹ and the CD2 molecule². Monoclonal antibodies directed at either CD2 or CD3 induce intracellular second messenger production and subsequent protein phosphorylation³⁻⁵. On most human non-B lymphocytes, CD3-Ti and CD2 are coexpressed and seem to be functionally interrelated⁶. But there are minor subpopulations in which these receptor systems can transduce signals despite a mutually exclusive expression^{7,8}, indicating that CD3-Ti and CD2 can act independently of each other. This view is supported by the finding that most monoclonal antibodies directed at the CD45 molecules^{9,10} are strongly co-mitogenic with CD2 but not CD3 monoclonal antibodies (refs 11, 12). As the intracytoplasmic domains of CD45 have tyrosine phosphatase activity¹³ these functional effects^{11,12} could be explained by a physical association between CD2 and CD45. Using chemical crosslinking techniques, we now show that CD45 is linked to CD2 on the surface of human T lymphocytes.

To investigate the influence of CD45 monoclonal antibody (mAb) on T-cell activation mediated by CD2 compared with CD3, we stimulated peripheral blood mononuclear cells (PBMC) either with mitogenic pairs of CD2 or CD3 mAbs (refs 14-16) in the presence or absence of a CD45 mAb (AICD45.1) (ref. 11). Independently of the combination of CD2 mAbs used, CD45 mAb strongly amplified the proliferative responses (Fig. 1a). By contrast, T-cell responses mediated through the CD3 molecule were somewhat inhibited, even when CD3 mAb was used in Sepharose-linked form (Fig. 1b). (Most CD45 mAbs do not influence CD3-mediated T-cell activation¹².) In contrast to the CD45 mAb that exerted differential activity on CD2 compared with CD3 activation, a CD28 mAb (9.3) (ref. 17) enhanced both CD3- and CD2-mediated T-cell responses (Fig. 1b). This suggests that CD28-mediated amplification is due to a mechanism that differs from the processes induced through CD45.

We investigated whether the above functional effects of CD45 mAb could be the result of a physical association between CD45 and CD2. To this end, co-precipitation experiments were carried out after chemical crosslinking of iodinated T-cell surface molecules by the homobifunctional crosslinker dithio-bissuccinimidylpropionate (DSP). After crosslinking, CD2 mAb precipitated—besides the typical 50K band—proteins of high relative molecular mass (M_r) which were very similar in mobility to the CD45 control immunoprecipitate (Fig. 2, lane B, upper

FIG. 1 Selective signalling of CD45 mAb on CD2-mediated human T-cell activation. *a*, Proliferative response of PBMC stimulated through CD2 in the presence (solid bars) or absence (stippled bars) of CD45 mAb (AICD45.1). *b*, Influences on CD3-mediated T-cell responses of CD45 mAb. Final concentrations of CD2 mAbs: anti-T11₂ and anti-T11₃ (ref. 2), 1:5,000 (v/v) (provided by Dr E. Reinherz); anti-T11_{1A} (AICD2.1.1A) (refs 11, 12), 1:400 (v/v). The CD2 mAbs D66 (ref. 14), VIT13 (ref. 15), 9-1 (ref. 16), YTH616.7 and the CD28 mAb 9.3 (ref. 17) were provided through the Fourth International Conference on Human Leucocyte Differentiation Antigens and used at a final dilution of 1:400 (v/v) of ascites fluid. CD45 mAb AICD45.1 (refs 11, 12) and CD28 mAb 9.3 were added at the onset of the cultures at a final dilution of 1:400 (v/v) of ascites fluid. CD3 mAbs: RW28CC8, 1:500 (v/v) (provided by Dr E. Reinherz); purified BMA 030, 0.01 $\mu\text{g ml}^{-1}$ (provided by Dr Kurre, Behring Werke); OKT-3, 1:32,000 (v/v). All mAbs were used in ascites form unless otherwise indicated. Purified OKT-3 was covalently bound to cyanogen bromide (CNBr)-activated Sepharose beads (S-OKT3) (ref. 23) and used at a final concentration of 1:160 (v/v) of packed beads.

METHODS. PBMC were prepared by Ficoll-Hypaque density centrifugation of heparinized whole blood. PBMC (5.0×10^4) were cultured in round-



bottomed microtitre plates in 200 μl RPMI 1640 medium, supplemented with 10% human serum, 1% penicillin-streptomycin and 2% glutamine. After stimulation, they were cultured for 72 h and, before collection, individual wells were pulsed for 16 h with 1 μCi ^3H -labelled thymidine ($185 \text{ GBq mmol}^{-1}$). Results represent means of triplicate cultures; s.d. <15%.

large arrow, compare C). Analogous results were obtained with several other CD2 mAbs (data not shown). In a reciprocal fashion, a 50K band (M_r 50,000) was visible in the precipitate obtained with CD45 mAb under crosslinking conditions (Fig. 2, D, lower large arrow).

If the high M_r proteins crosslinked to CD2 were the CD45 molecules, then the CD2 immunoprecipitate should contain phosphatase activity. A CD2 immunoprecipitate prepared from DSP-treated T cells exerted strong enzymatic activity towards the synthetic phosphatase substrate *p*NPP (Fig. 3a). By contrast, CD27 mAb co-precipitated only low levels of phosphatase activity. As CD27 is expressed in similar quantity to CD2 on the surface of resting T lymphocytes, this experiment clearly excludes the possibility of a nonspecific co-precipitation of phosphatase activity by CD2 mAb. The phosphatase co-precipitated by CD2 mAb under crosslinking conditions probably belongs to the protein tyrosine phosphatases, as it was inhibited by micromolar concentrations of sodium vanadate. Also, it did not show any activity in the absence of sulphhydryl components¹⁸

(data not shown). More importantly, depletion of CD45 resulted in loss of the phosphatase activity co-precipitated by CD2 mAb (Fig. 3a).

We confirmed the association between CD2 and CD45 by using a solid phase enzyme-linked immunosorbent assay (ELISA) system (Fig. 3b). There, CD45 was specifically detected in a CD2 immunoprecipitate obtained from DSP-treated T lymphocytes. The decrease in the amount of CD45 detectable in the ELISA system at higher concentrations of DSP results from a loss of reactivity of the CD2 molecule with the respective mAb (data not shown). The CD2-CD45 association was also observed on the T lymphoma cell line Jurkat, in which CD2 mAb exclusively co-precipitated the CD45 molecules and vice versa (Fig. 3c). The CD2-CD45 complex was found to be formed between 0.3 nm and 1.6 nm on human T lymphocytes, as judged from an experiment in which T lymphocytes were treated with cross-linkers spanning different distances before immunoprecipitation with CD2 mAb (Fig. 3d).

The high M_r molecules co-precipitated by CD2 mAb were

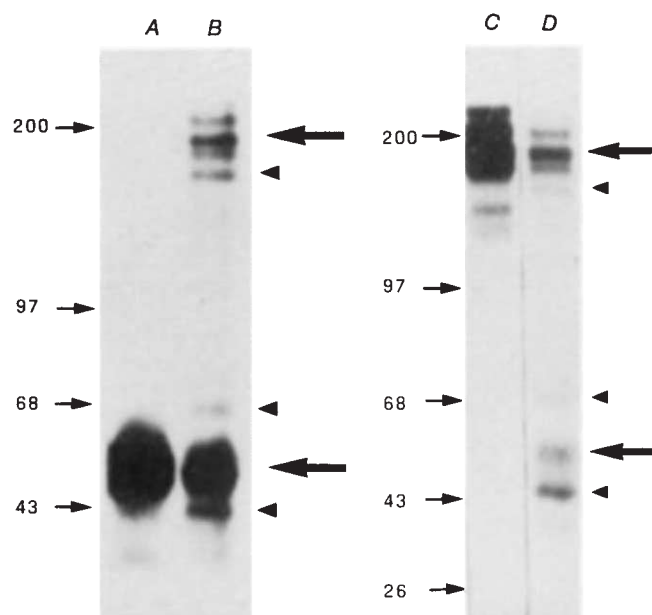
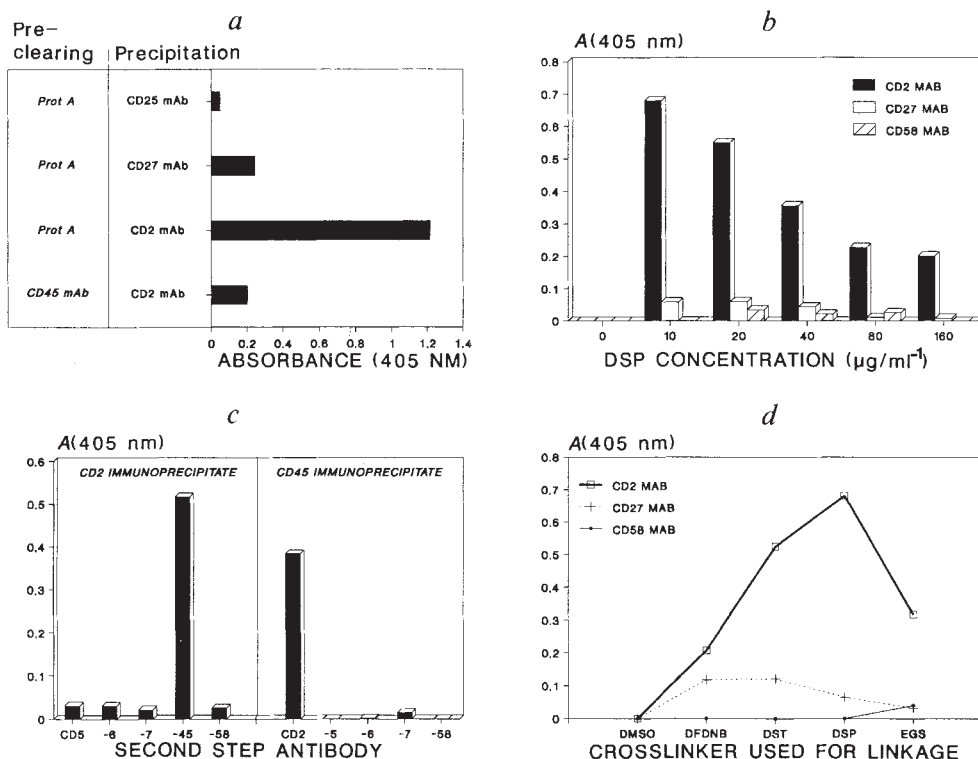


FIG. 2 Immunoprecipitation of ^{125}I -labelled T-cell surface molecules showing preferential association between CD2 and high M_r proteins after chemical crosslinking. Autoradiography of immunoprecipitates using CD2 mAb (3PT2H9, provided by Dr E. Reinherz, lanes A, B) and CD45 mAb (AICD45.2, lanes C, D). Mock crosslinked immunoprecipitates are shown in lanes A, (CD2) and C (CD45); those obtained under crosslinking conditions are shown in lanes B (CD2) and D (CD45). Large arrows indicate the typical CD45 and CD2 molecules. Small arrows indicate material which co-precipitated with CD2 and CD45 after crosslinking. Differences in the migration distances of precipitated proteins result from the experimental conditions, as the CD2 precipitates were run on a separate gel to avoid contamination from CD45 precipitates. Number, M_r 's ($M_r \times 10^{-3}$).

METHODS. PBMC were enriched for T lymphocytes by E-rosette formation followed by Ficoll-Hypaque density centrifugation and subsequent lysis of SRBC in hypotonic buffer. Cells were incubated overnight at 37 $^{\circ}\text{C}$, 7% CO_2 and 100% humidity to allow depletion of adherent cells. Nonadherent cells (>95% reactive with CD2 mAb) were collected, washed twice with PBS, pH 7.4, and labelled with ^{125}I (1 mCi per 4×10^7 lymphocytes) by the glucose oxidase technique. Chemical crosslinking was performed in PBS, pH 8.3, at a density of 5×10^6 cells ml^{-1} and 50 $\mu\text{g ml}^{-1}$ 3,3'-DSP dissolved in dimethyl sulphoxide (DMSO). For mock crosslinking, DMSO was used without DSP. After crosslinking, cells were washed twice in PBS, pH 7.4, and then lysed in 400 μl RIPA buffer containing 1% Triton X-100, 0.15 M NaCl, 0.5% sodium desoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM iodoacetamide and 2 $\mu\text{g ml}^{-1}$ trypsin inhibitor. Antigens were immunoprecipitated from precleared lysates using monoclonal antibodies covalently bound to CNBr-activated Sepharose 4B. Washed precipitates were then subjected to SDS-PAGE on gradient slab gels (5-15%) under reducing conditions.

FIG. 3 *a*, Enzymatic activity of immunoprecipitates obtained from Triton X-100 lysates of crosslinked T lymphocytes towards the synthetic phosphatase substrate *p*-nitrophenyl phosphate (*p*NPP). Antibodies used for immunoprecipitation were CD27 mAb (AICD27.1) and CD2 mAb (ICRFCD2.1). Negative control was CD25 mAb (AICD25.1). *b*, Identification of CD45 co-precipitated by CD2 mAb under crosslinking conditions by a solid phase ELISA system. *c*, Association between CD2 and CD45 on the T-lymphoma cell line Jurkat. *d*, Influence of crosslinkers spanning different distances on the amount of CD45 co-precipitated by CD2 mAb.

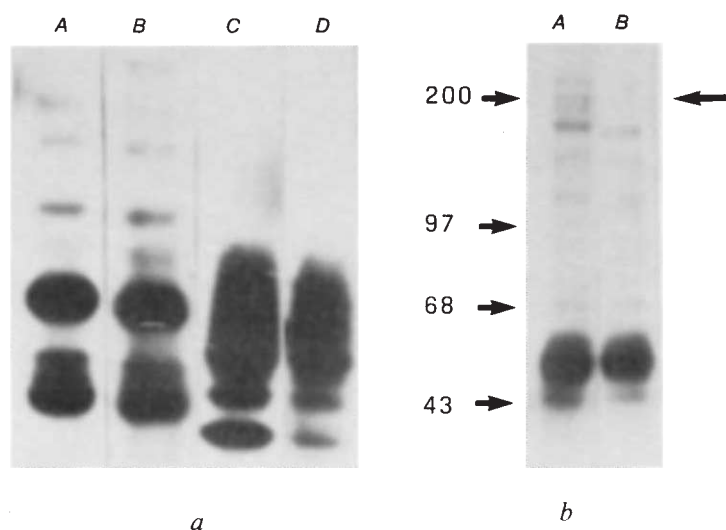
METHODS. *a*, ^{125}I -labelled T lymphocytes treated with $12.5 \mu\text{g ml}^{-1}$ DSP were lysed in buffer: 150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, pH 7.2. Immunoprecipitation was carried out as in Fig. 2. Washed immunoprecipitates (1.4×10^5 c.p.m.) were incubated for 5 h at 37°C with 200 μl substrate buffer: 50 mM HEPES, 100 mM KCl, 3 mg ml^{-1} DTT, 1 mg ml^{-1} *p*NPP, 0.1% Triton X-100, pH 6.9. Beads were spun down and absorbance of the 100 μl supernatant determined at $\lambda = 405$ nm using a Titertek Multiscan Analyser. Left panel: Lysates of DSP-treated T lymphocytes were pre-cleared six times with CD45 mAb before immunoprecipitation with CD2 mAb. *b*, T lymphocytes were treated with either DMSO or DSP at the concentrations indicated. Immunoprecipitation was performed in 96-well microtitre ELISA plates coated with either 1 μg CD2 mAb (ICRFCD2.1, IgG2a) or 1 μg CD27 mAb (AICD27.1, IgG2a). CD58 mAb (AICD58.1, IgG2a), which is unreactive with resting T lymphocytes, was used as a negative control. Lysates (5×10^6 cells per well) were incubated for 2 h at room temperature. After immunoprecipitation, plates were washed four times with 200 μl lysis buffer, and immunoprecipitates incubated for 1 h with 100 μl CD45 mAb (AICD45.2, IgG1, $10 \mu\text{g ml}^{-1}$ in PBS-1% BSA). Plates were then washed three times with 200 μl PBS-1% BSA. Alkaline phosphatase-labelled goat anti-mouse IgG1 (100 μl) (1:400 (v/v) in PBS-1% BSA) was added and incubated for 1 h. Finally, plates were washed four times with PBS-1% BSA, and 100 μl substrate buffer (50 mM HEPES, 100 mM KCl, 1 mg ml^{-1} *p*NPP, pH 9.0) was added. Absorbance was determined as above. Results are means of dupli-



cates. *c*, Jurkat cells were treated with DSP ($12.5 \mu\text{g ml}^{-1}$) before lysis and immunoprecipitation. Lysates were added to 96-well microtitre plates coated with 1 μg mAbs to CD45 (AICD45.2, IgG1) or CD2 (ICRFCD2.1, IgG2a). To identify co-precipitated proteins, 100 μl mAb directed at the indicated surface molecule was added at a 1:400 dilution of ascites fluid. Binding of second-step mAb was then determined with alkaline phosphatase-conjugated goat anti-mouse antiserum specifically directed at the mAb isotype. Mabs: CD5 (C1.1E4.7, IgG1, MF7-14-15, IgG2a), CD6 (M-T603, IgG1, MT-605, IgG2a), CD7 (F101-229, IgG1, PANL/D, IgG2a) (Fourth International Conference on Human Leucocyte Differentiation Antigens), CD58 mAb (TS2/9, IgG1), and CD58 mAb AICD58.1 (IgG2a). Results are means of duplicates. *d*, T lymphocytes were treated with crosslinkers before lysis and immunoprecipitation. DFDNB, 1,5-difluoro-2,4-dinitrobenzene (0.3 nm, $12.5 \mu\text{g ml}^{-1}$); DST, disuccinimidyl tartrate (0.64 nm, $100 \mu\text{g ml}^{-1}$); DSP (1.2 nm, $12.5 \mu\text{g ml}^{-1}$); EGS, ethylene glycolbissuccinimidylsuccinate (1.61 nm, $12.5 \mu\text{g ml}^{-1}$). Immunoprecipitation as described in *b*.

FIG. 4 Identification of the proteins co-precipitated by CD2 and CD45 mAb under crosslinking conditions. V8 protease digests of the directly immunoprecipitated CD45 and CD2 molecules and the proteins co-precipitated by CD45 and CD2 mAbs. Lane A, Peptide pattern of the directly precipitated CD45 molecules (see Fig. 2, lane C). Lane B, Proteins co-precipitated by CD2 mAb (upper arrows in Fig. 2, lane B). Lane C, Cleavage fragments of the CD2 molecule (see Fig. 2, lane A). Lane D, Protein co-precipitated by AICD45.2 (lower arrow Fig. 2, lane D). *b*, Loss of the high *M_r* proteins in the CD2 immunoprecipitate after depletion of CD45. Lane A, CD2 immunoprecipitate from a CD27 (AICD27.1)-depleted lysate. Lane B, CD2 immunoprecipitate from a CD45 (AICD45.2)-depleted lysate.

METHODS. *a*, Peptide mapping was performed using a modification of the method of Cleveland *et al.*¹⁹. After autoradiography, proteins were excised from dried and unfixed gels. Gel slices were rehydrated for 15 min in 100 μl SDS sample buffer (10% glycerol, 2% SDS, 0.063 M Tris buffer, pH 6.8, 0.01% bromophenol blue, 0.05 M DTT) containing 0.1 mg ml^{-1} V8 Protease (endoproteinase Glu-C). Reswollen slices were placed in the sample well of a 15–22.5% SDS-PAGE and digested with V8 protease for another 30 min. Electrophoresis was started at 50 V until samples had completely migrated into the stacking gel, then at 70 V with an interruption for 30 min shortly before samples had reached the separating gel, and then continued under standard conditions. Peptides were visualized by autoradiography of dried gels. *b*, T lymphocytes were labelled, crosslinked and lysed as described in Fig. 2. Lysates were pre-cleared six times with CD45 mAb (AICD45.2) or CD27 mAb before immunoprecipitation with CD2 mAb (3PT2H9). Numbers, *M_r*, (*M_r* $\times 10^{-3}$).



further investigated by peptide mapping using V8 protease treatment¹⁹. After digestion, the 180K–220K proteins in the CD2 immunoprecipitate (Fig. 4a, lane B) produced peptide patterns identical to those of the directly immunoprecipitated CD45 molecules (Fig. 4a, lane A). Formal proof that these proteins are indeed CD45 was provided by the pre-clearing experiment shown in Fig. 4b. Depletion of CD45 resulted in complete loss of the four high M_r bands in the CD2 immunoprecipitate (Fig. 4b, lane B). That CD2 could be precipitated from the CD45-depleted lysate indicates that not all CD2 molecules are linked to CD45.

In an analogous way, the 50K protein co-precipitated by CD45 mAb (Fig. 4a, lane D) was identical on V8 protease treatment to the directly precipitated and digested CD2 molecule (Fig. 4a, lane C). Moreover, the CD2–CD45 molecular complex could specifically be reprecipitated from a CD45 immunoprecipitate by CD2 mAb, indicating that the 50K protein which was present in the crosslinked CD45 immunoprecipitate was indeed CD2 (data not shown). It should be noted that CD2–CD45 association also exists on murine T lymphocytes²⁴.

Recent results have shown that artificial bringing together of CD45 to other triggering receptors (CD2 and CD3-Ti) on the T-cell surface by means of mAb heteroconjugates downregulates their capacity for signal transduction²⁰. The homology between the cytoplasmic domains of CD45 and a tyrosine phosphatase of human placenta^{18,21} could help to interpret the observed functional effects of CD45 mAbs. Thus, a physiological mechanism of 'negative signalling' and downregulation of CD2-mediated T-cell activation could exist based on the preferential steric association between CD2 and CD45. Binding of monoclonal antibody to CD45 could alter the localization of the CD45 phosphatase in relation to CD2 and produce changes in phosphorylation–dephosphorylation events involved in signal transduction. Enhancement of CD2-dependent T-cell activation by CD45 mAbs could then result from inhibition of physiological CD2–CD45 interactions, preventing or slowing down protein dephosphorylation. Alternatively, CD45 mAbs could alter the phosphatase activity of CD45. These possibilities need to be elucidated, particularly as individual isoforms of CD45 gene products seem to behave in a functionally different manner after mAb binding^{11,12}.

Three additional molecules were found in the CD2–CD45 molecular complex (Fig. 2, lanes B and D, triangles). Whereas the nature and function of the 150K and the 65K molecules are unclear at present, peptide map analysis demonstrated that the 43K protein co-precipitated by CD2 mAb and CD45 mAb represents the human major histocompatibility complex (MHC) class I protein (not shown). Given recent findings indicating that monoclonal antibodies (like CD45 mAbs) reactive with MHC class I molecules selectively modulate T-cell activation through CD2 (ref. 22), this is highly intriguing.

Whether CD2-mediated T-cell activation represents a truly 'alternative pathway' *in vivo*, and whether it is dependent or independent of the T-cell antigen receptor (TCR) complex, remains to be examined further. Nevertheless, the present data indicate that because of the preferential association between CD2 and CD45, this pathway of T-cell activation could be regulated independently of the TCR complex. More importantly, external engagement of the transmembrane CD45 molecules could modulate the signalling strength of CD2-dependent stimuli at the intracellular level of protein phosphorylation. Such a mechanism of finely tuning receptor function may provide a means by which T-cell reactivity can be adjusted to particular environmental requirements *in vivo*. □

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The B-cell antigen CD22 mediates monocyte and erythrocyte adhesion

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INTERACTION with antigen-presenting accessory cells is thought to be an important step in B-cell activation^{1–5}, and the B-cell receptor CD22, which is coordinately expressed with surface immunoglobulin, has been proposed to participate in the antigen response⁶. Here we show that CD22 has a structure closely related to myelin-associated glycoprotein (MAG, a neuronal adhesion protein), and mediates monocyte and erythrocyte adhesion. Like CD2, the T-cell erythrocyte receptor, CD22 may facilitate antigen recognition by promoting antigen-nonspecific contacts with accessory cells.

B cells respond to antigen primarily in the lymphoid organs (lymph nodes, Peyer's patches and spleen) in which antigen is concentrated and presented by accessory cells³. The physical association between these cells and responding B lymphocytes predicts the latter may express surface molecules similar to the T-cell molecules (such as CD2, CD4 and CD8) which promote T cell–target cell interaction. The B cell-specific receptor CD22 shares several distinctive features with these T cell structures, including developmental specificity, the capacity to mediate antigen-nonspecific adhesion, and a potential role in the facilitation of antigen recognition. As with these other molecules, expression of CD22 closely follows that of the antigen receptor. Both IgM and CD22 are expressed in the cytoplasm but not on the surface of pre-B cells and early B acute lymphoblastic leukaemias (B-ALL)⁷. CD22 appears on the cell surface shortly after IgM and at about the same time that IgD appears^{6,7}. Like surface IgD, CD22 expression is lost when B cells are stimulated with anti-immunoglobulin antibody or protein A^{6,7}. CD22-negative, IgM-positive B cells do not respond to anti-IgM with an increase in cytosolic free calcium, whereas CD22-positive, IgM-positive B cells readily so respond⁶.

To isolate a CD22 complementary DNA, an expression library was constructed from the Burkitt lymphoma cell line Daudi,

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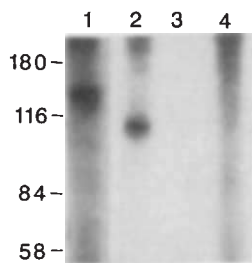


FIG. 1 Immunoprecipitation of CD22 from transfectants and cell lines: lane 1, Burkitt lymphoma Raji cells; lane 2, transfected COS cells; lane 3, Raji cells with a control isotype-matched antibody; lane 4, transfected COS cells with a control isotype-matched antibody. Molecular weights ($\times 10^{-3}$) are indicated on the left.

METHODS. COS cells were transfected and labelled with ^{125}I as described^{8,9}, washed with PBS buffer and lysed with a buffer containing 1% Nonidet-P40, 50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl_2 , 5 mM KCl and 1 mM phenylmethylsulphonyl fluoride (PMSF). The lysates were centrifuged, precleared with 10 μg of isotype-matched mouse IgG and protein A beads (Pierce) at 4 °C overnight, and incubated with 10 μg Leu-14 monoclonal antibody (mAb) and fresh protein A beads (4 h, 4 °C). The beads were washed 3 times in lysis buffer, resuspended in loading buffer^{8,9} containing mercaptoethanol and eluted by boiling. Eluates were electrophoresed on a 7% polyacrylamide gel and the dried gel autoradiographed for 24 h.

introduced into COS cells by the DEAE-dextran method and subjected to three rounds of panning and re-introduction into *Escherichia coli* as described^{8,9}. Of 16 plasmids picked after the third round, two tested positive for CD22 expression by indirect immunofluorescence in COS cells. Of the five carbohydrate-related epitopes recognized by anti-CD22 monoclonal anti-

bodies^{10,11}, only two (epitopes A and D) were expressed in COS cells.

Immunoprecipitation of CD22 from transfected COS cells yielded a single band corresponding to a relative molecular mass (M_r) of 110,000 (110K), smaller than the 135K species obtained from Raji cells (Fig. 1). A similar diminution of the mass of surface antigens expressed in COS cells has previously been noted^{8,9}, and suggested to be related to differences in glycosylation. As the immunogenic epitopes of CD22 are carbohydrate-related, these differences might account for the absence of epitopes B, C and E.

Northern blot analysis revealed the presence of a major 3-kilobase (kb) RNA species and four minor species of 2.6, 2.3, 2.0 and 1.5 kb (Fig. 2a). Message abundance was highest in the Raji Burkitt lymphoma line followed by the B lymphoblastoid line IM-9 and the EBV-transformed line CESS. No transcripts were found in the plasmacytoma RPMI 8226, consistent with previous observations that CD22 is lost as B cells differentiate to plasma cells^{6,7}. Little RNA was found in the pre-B line Nalm-6, which expresses cytoplasmic but not surface CD22 (ref. 7), and no RNA was observed in non-B cells including peripheral blood T cells, the T-cell leukaemia cell line Jurkat, the myeloid leukaemia lines HL60 and U937 and the hepatoma cell line HepG2.

DNA blot hybridization of placental DNA gave a simple pattern consistent with a single copy gene (Fig. 2b). Analysis of B-cell DNAs digested with *EcoRI*, *HindIII* and *BamHI* restriction endonucleases showed no evidence for CD22 gene rearrangement during B-cell development (data not shown).

DNA sequence analysis by the dideoxy chain termination method showed that the 2,116-bp insert encoded a polypeptide of 647 amino acids with a translational initiation sequence similar to the known consensus¹² (Fig. 3a). The amino-terminal methionine is followed by 18 predominantly hydrophobic amino

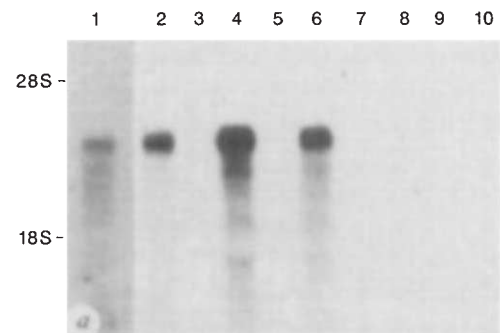


FIG. 2 Characterization of the clone. *a*, RNA blot hybridization analysis of CD22 transcripts. Total RNA (20 μg) was denatured, electrophoresed through agarose, transferred to nylon membranes, hybridized with ^{32}P -labelled CD22 cDNA and exposed for either 72 h (lane 1) or 24 h (lanes 2–10). RNA sources were: lane 1, Nalm-6 (pre-B cell leukaemia); lane 2, IM-9 (B lymphoblastoid cell line); lane 3, peripheral blood T cells; lane 4, Raji (Burkitt lymphoma); lane 5, Jurkat (T cell leukaemia); lane 6, CESS (EBV-transformed B lymphoblastoid cell line); lane 7, U937 (histiocytic lymphoma); lane 8, RPMI 8226 (plasmacytoma); lane 9, HL60 (promyelocytic leukaemia); and lane 10, HepG2 (hepatoma). Migration of 28S and 18S rRNA markers is indicated. *b*, DNA blot hybridization analysis of CD22 genomic DNA. Human placental DNA (20 μg) were digested with restriction endonucleases, electrophoresed through agarose, transferred to nylon, hybridized and exposed for 48 h. The restriction enzymes used were: lane 1, *EcoRI*; lane 2, *BamHI*; lane 3, *HindIII*; and lane 4, *DraI*.

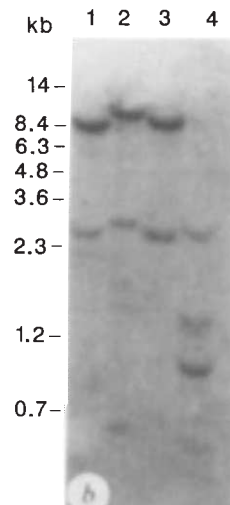
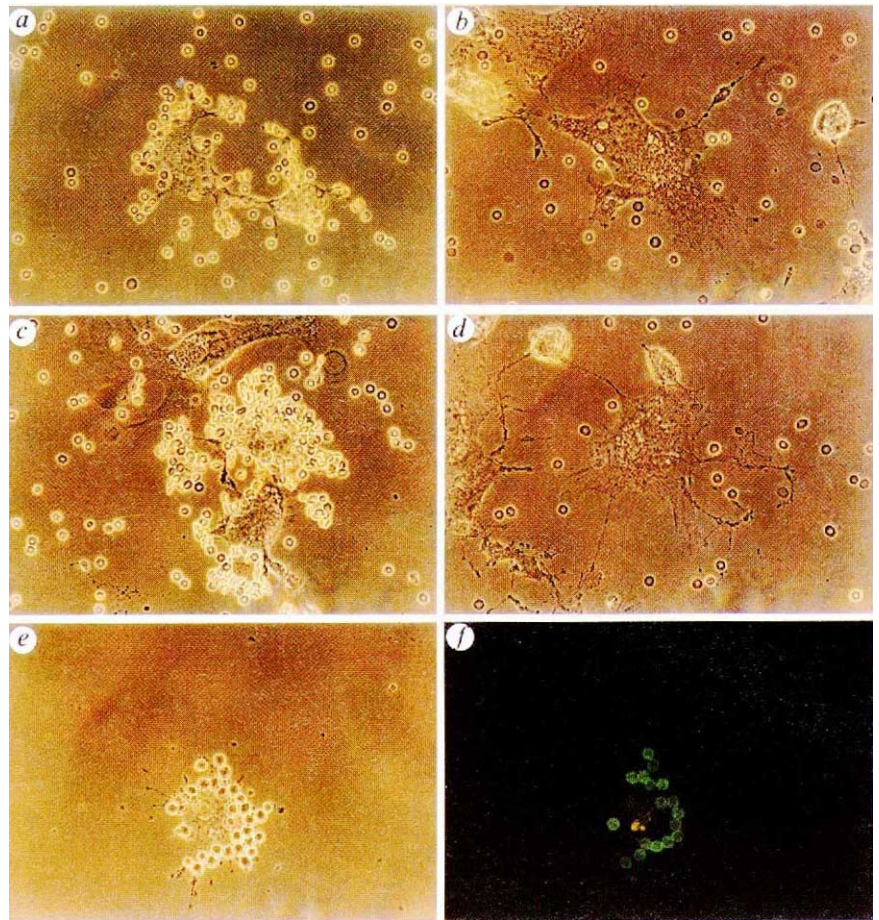


FIG. 4 CD22 mediated erythrocyte and monocyte adhesion. *a-d*, Binding of erythrocytes to *a*, transfected COS cells; *b*, transfected COS cells pre-incubated with anti-epitope A mAb; *c*, transfected COS cells pre-incubated with anti-epitope D mAb; *d*, COS cells transfected with an unrelated cDNA. *e, f*, Mononuclear cell rosettes (treated with fluorescein-conjugated anti-CD14 mAb) with CD22 transfected COS cells shown under *e*, phase contrast or *f*, fluorescence illumination.

METHODS. CD22 mAb were obtained from the Fourth International Leukocyte Typing Workshop. Leu-3 (anti-CD4), Leu-M3 (anti-CD14) and Leu-16 (anti-CD20) mAb were purchased from Becton Dickinson (Mountainview, CA). Peripheral blood mononuclear cells were separated on Ficoll/diatrizoate gradients, washed several times with PBS and resuspended in Dulbecco's modified Eagle's medium (DMEM) in the presence of heparin (500 U ml^{-1}). COS cells transfected with CD22 or a control cDNA clone were trypsinized a day after transfection and cultured in 6-cm dishes for 1–2 more days, then overlaid with mononuclear cells or erythrocytes in 2 ml of DMEM with 500 U ml^{-1} heparin and incubated at 4°C for 1 h with gentle rocking. Non-adhering cells were removed by gentle washing with PBS and the remaining cells were stained with fluorescein labelled anti-CD4 (Leu-3), anti-CD14 (Leu-M3), or anti-CD20 (Leu-16) (Becton Dickinson), for 30 min at 22°C , washed with PBS, fixed in 4% formaldehyde and examined by fluorescence microscopy. Monoclonal antibody-blocking studies were conducted by preincubating CD22 transfectants with anti-epitope A (Leu-14) or anti-epitope D (BL-3C4) mAb for 30 min at 22°C . Adhesion assays were carried out as above. The experiments were performed in DMEM, although similar results were obtained in PBS without calcium or magnesium and in the presence of 0.02% sodium azide.



Rosettes with mononuclear cells were stained with the lineage-specific antibodies anti-CD14, anti-CD4 and anti-CD20; most rosetting cells reacted with anti-CD14, which recognizes monocytes (Fig. 4). Preincubation of COS-cell transfectants with either anti-epitope A or anti-epitope D antibody failed to block mononuclear cell adhesion, but when both antibodies were used in conjunction, partial blocking was observed (data not shown). These results suggest that different epitopes of CD22 participate in erythrocyte and monocyte adhesion and that different ligands may be recognized on each cell type.

Expression of the B-cell erythrocyte receptor^{21–24} defines a subpopulation of small mature B cells which respond to T cell-independent antigens²⁵, and which is similar, if not identical, to the CD22-positive sub-population. Like CD2, the T-cell erythrocyte receptor, CD22 may facilitate antigen recognition by promoting accessory cell adhesion.

Within the T-cell lineage, the CD2, CD4 and CD8 molecules associate physically and developmentally with the antigen receptor, and are each thought to promote antigen recognition through antigen-nonspecific contacts with target cells. The present findings raise the possibility that CD22 may similarly promote recognition by the B-cell antigen receptor by intensifying B cell-presenting cell contacts. CD22 has previously been implicated in the transmission of signals synergizing with the antigen receptor²⁶; and crosslinking of surface IgM produces an intracellular calcium flux in $\text{IgM}^+\text{CD22}^+$ but not in $\text{IgM}^+\text{CD22}^-$ cells⁶. These results suggest that, like the T cell accessory molecules, CD22 may also participate in the regulation of signal transduction.

Note added in proof: G. L. Shen *et al.*²⁷ have reported that the NALM-6 pre-B cell line expresses both surface and cytoplasmic CD22, consistent with the transcripts we find in these cells. □

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A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting

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GROWTH factors are thought to function as pivotal autocrine-paracrine regulatory signals during embryonic development^{1,2}. Insulin-like growth factor II (IGF-II), a mitogenic polypeptide for a variety of cell lines^{3,4}, could have such a role, as indicated by the pattern of expression of its gene during rodent development. The IGF-II gene⁵⁻⁷ uses at least three promoters^{5,8,9} and expresses several transcripts in many tissues during the embryonic and neonatal periods^{5,10}, whereas expression in adult animals is confined to the choroid plexus and the leptomeninges¹¹. To examine the developmental role of IGF-II, we have begun to study the consequences of introducing mutations at the IGF-II gene locus in the mouse germ line. We have disrupted one of the IGF-II alleles in cultured mouse embryonic stem (ES) cells¹²⁻¹⁴ by gene targeting¹⁵⁻¹⁸ and constructed chimaeric animals. Germ-line transmission of the inactivated IGF-II gene from male chimaeras yielded heterozygous progeny that were smaller than their ES cell-derived wild-type littermates (about 60% of normal body weight). These growth-deficient animals were otherwise apparently normal and fertile. The effect of the mutation was exerted during the embryonic period. These results provide the first direct evidence for a physiological role of IGF-II in embryonic growth.

Various strategies of gene targeting (homologous recombination between an introduced mutated gene and an endogenous chromosomal allele) have been used to disrupt genes in ES cells^{16,17,19-25}, and some of the targeted clones have been used successfully to generate germ-line chimaeras^{24,25}. To target the IGF-II gene, we used a replacement vector containing two selectable markers, the bacterial neomycin-resistance gene (*neo*) and the herpes simplex virus (HSV) thymidine kinase gene (*tk*)¹⁷. The markers allow the application of a positive-negative selection protocol¹⁷, using the drugs G418 and gancyclovir (GANC), that identifies DNA recipient cells while lowering the background of random integrants, resulting in significant enrichment for targeted clones (homologous recombinants). In our experiments, the use of GANC for negative selection was not detrimental for the pluripotency of the ES cells; a targeted ES cell line obtained by following this strategy was used successfully for the generation of germ-line chimaeras.

For the targeting procedure, we first isolated a phage λ clone carrying the mouse IGF-II gene (Fig. 1A). The organization of this gene is very similar to that of the rat homologue that we have characterized extensively^{5,8,10,11}. Fragments of the cloned IGF-II gene and transcriptionally competent *neo* and *tk* cassettes^{16,17} were used for the construction of the replacement vector (Fig. 1A). The *neo* cassette, replacing 0.25 kilobases (kb) of IGF-II gene sequence in the region of the first coding exon (E2), was flanked by 1.6 and 8.3 kb of mouse IGF-II locus DNA (5' and 3', respectively). The *tk* cassette was attached at the 3' end of the 8.3-kb fragment (Fig. 1A). Successful targeting with this vector will abolish the function of the IGF-II gene; in primary transcripts from the intact IGF-II promoters, processing should occur at the polyadenylation site of the *neo* gene, whereas any products that might arise from abnormal splicing, if able to be translated, would not yield a functional polypeptide.

Linearized replacement vector DNA was introduced into recipient CP1 or CCE ES cells^{26,27} by electroporation, and the

TABLE 1 Frequency of homologous recombination after double drug selection

ES cell line	Cells electroporated $\times 10^7$	G418 ^R colonies	G418 ^R /GANC ^R colonies (% of G418 ^R colonies)	Homologous recombinants (% of G418 ^R /GANC ^R colonies)
CP1				
Expt I a	4	200		
b	20		94 (9.4)	
Expt II	22		214	7 (3.3)
Expt III a	2	36		
b	26		42 (9)	
CCE				
Expt IV a	1	63		
b	1		5 (7.9)	
Expt V	28		54	1 (1.9)
Expt VI a	2	90		
b	25		40 (3.6)	1 (2.5)

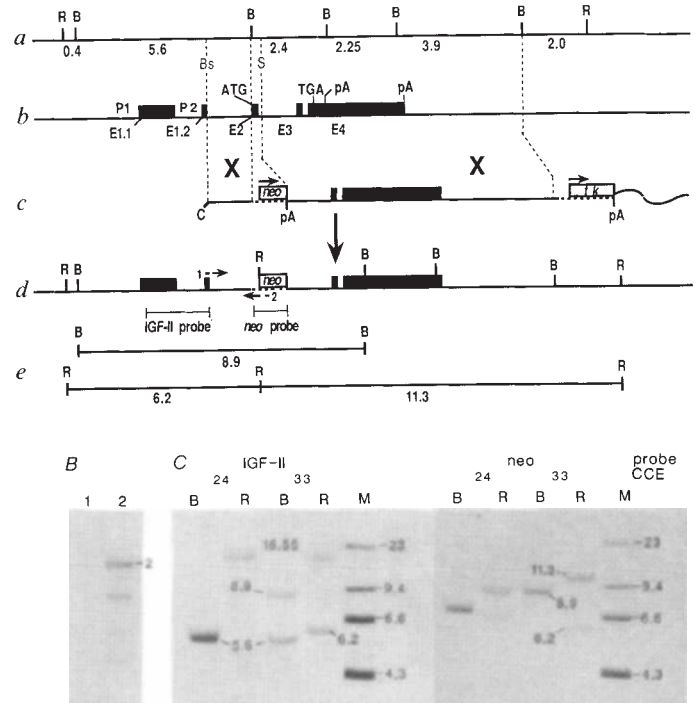
ES cells were grown to 60–70% confluence as described²⁸ on mitomycin C-treated G418-resistant STO feeder layers. Cells at passage 10 (2×10^7 cells in 0.5 ml 20 mM HEPES buffer, pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM dextrose, and 0.1 mM 2-mercaptoethanol) were electroporated with 25 μg *Cla*I-linearized replacement vector DNA (Fig. 1A) in a 0.4-cm cuvette using a BioRad Gene Pulser at 220 V (capacitance, 960 μF), and then plated at 2×10^7 cells per 10-cm feeder plate. G418 (225 $\mu\text{g ml}^{-1}$; effective concentration) was added 48 h after plating. GANC (2 μM) was also added 72 h later to some of the plates (experiments II and V, and the 'b' series of the other experiments). The cells in the 'a' series of plates were maintained under G418 only. In experiments III–VI, the cells were not split before adding the drug. After selection for 14 days, drug-resistant colonies were picked and expanded (without selection) for further analysis. To identify targeted clones, DNA from colony pools (experiment II) or individual colonies (experiments V and VI) was assayed by PCR (Fig. 1B). The percentage of G418-resistant colonies that were also resistant to GANC was calculated by extrapolation from the results of the 'a' and 'b' series of experiments. Successful targeting in the PCR-positive pools or individual colonies was verified by Southern-blot analysis. The targeting events scored from assays of colony pools (experiment II) are independent because they correspond to independent electroporations. G418^R and GANC^R, G418-resistant and GANC-resistant, respectively.

cells were seeded and selected on feeder layers of STO fibroblasts²⁸. In a series of experiments (Table 1), we obtained similar results with both recipient lines; on average, $7.5 \pm 2\%$ of the G418-resistant colonies were also resistant to GANC, and of these, $2.6 \pm 0.6\%$ were homologous recombinants, as determined by polymerase chain reaction (PCR) assays (Fig. 1B). Successful targeting occurred, although the IGF-II gene is not transcribed in undifferentiated ES cells at levels detectable by northern-blot analysis (data not shown). Detectable expression of a gene in ES cells is not a prerequisite for its successful targeting²².

From one of the experiments with CCE cells (experiment VI, Table 1), we identified a targeted clone by assaying individual colonies by PCR. This clone, which was recovered rapidly without extensive culturing and/or sib-selection from colony pools, was expanded into a cell line (CCE.33). The disruption of the IGF-II gene in this line and the absence of additional random copies of the replacement vector were verified by Southern-blot analysis (Fig. 1C).

We injected CCE.33 cells into host blastocysts obtained from the mouse strains CD-1, MF1 and C57Bl/6. The first two strains are albino (homozygous for the *c* allele), whereas the third strain is black, nonagouti (*BB aa*). As the CCE cells were derived from a blastocyst of the 129/Sv/Ev strain²⁷, which is black, agouti (*BB AA*), chimaeric mice could be scored by the presence of pigmented coat hair in mice from the CD-1 and MF1 blastocysts, and by the presence of agouti hair in animals of C57Bl/6 genetic background. The frequency of formation of overt chimaeras was high with all three strains of blastocysts (25–54%; Table 2a). As judged from the CCE.33 cell contribution to coat colour, however, the degree of chimaerism varied depending on the genetic background of the host blastocyst, in agreement with previous results²⁵. Therefore, the contribution of CCE.33 cells was extensive in combination with the C57Bl/6 genotype, only occasionally extensive in the MF1 background, and poor in the progeny of CD-1 blastocysts.

FIG. 1 **A**, Targeting scheme for disruption of the mouse IGF-II gene. **a**, *EcoRI* (R) and *BamHI* (B) sites and the distances (in kb) between these sites are indicated in a restriction map of the IGF-II locus, derived from the analysis of a λ phage clone (IGF-II.3.1) in conjunction with genomic blotting. This clone was isolated by screening a mouse genomic library (provided by K. Signorelli) with a rat IGF-II coding region probe¹⁰. This library was constructed by cloning into the EMBL3 vector chromosomal DNA fragments generated by partial *Sau3A* digestion. **b**, The positions of five exons (E1.1–E4; black boxes) relative to the map in **a** are indicated. P1 and P2 denote the regions of two promoters generating the alternate 5' noncoding exons E1.1 and E1.2. The positions of the initiation codon (ATG), termination codon (TGA), and two alternative polyadenylation sites (pA) are shown. **c**, Diagram of the replacement vector (linearized at a *Clal* site; C) containing the *neo* and *tk* cassettes, each driven by a mutant polyoma virus enhancer and the HSV *tk* promoter^{16,17}. The *neo* cassette (containing a single *EcoRI* site) is a blunt-ended 1.15-kb *XhoI*-*BamHI* fragment from plasmid pMC1 pola (Stratagene; a derivative of pMC1neo¹⁶ containing a polyadenylation signal) that was ligated to the 3' end of a blunt-ended 1.6 kb IGF-II fragment extending from a *BstE* II (Bs) site at the end of exon E1.2 to a (destroyed) *BamHI* site immediately downstream of the ATG codon in E2. The rest of E2 and about 0.1 kb of the downstream intron (a 0.25-kb *BamHI*-*SmaI* fragment) has been replaced by the *neo* cassette. The 3' end of this cassette was connected to an 8.3 kb IGF-II fragment (beginning at the intron *SmaI* site; S). The *tk* cassette (a 1.85 kb fragment from plasmid pMC1tk, provided by K. Thomas and M. Capecchi) was ligated at the end of the 8.3 kb fragment. The rest of the vector (pIC20R; 2.7 kb)²⁹ is shown as a wavy line. **d**, Predicted structure of the targeted IGF-II locus. Two dashes (1 and 2) indicate the positions of the synthetic oligonucleotide primers (20 residues each) that were used for PCR assays (**B**), performed as described (ref. 30). The 5' end of primer 1 (5'-CATCCTCTTCCAGCCCCAGC-3') corresponds to position 41 upstream from the 5' splice site of E1.2. The 5' end of primer 2 (5'-TGCGCTGACAGCCGGAACAC-3') corresponds to position 415 from the beginning of the *neo* cassette. The locations of the IGF-II and *neo* probes, used for Southern-blot analysis (**C**), are indicated. **e**, Predicted sizes of targeted DNA fragments that can be detected by the probes. **B**, Examples of negative (lane 1) and positive (lane 2) PCR assays using the primers 1 and 2 (**A**) and DNA extracted from pools of colonies resistant to both G418 and GANC. The amplified DNA of predicted size (2 kb) and some partial products were visualized after Southern transfer using a 0.6 kb probe prepared from the intron between E1.2 and E2. **C**, Southern analysis of



BamHI- or *EcoRI*-digested genomic DNA from the targeted cell line CCE.33 and a random integrant (CCE.24). The membrane was hybridized to the IGF-II probe, then stripped and rehybridized to the *neo* probe, as indicated. The IGF-II probe hybridizes to the wild-type IGF-II 5.6-kb *BamHI* and 16.55 *EcoRI* DNA fragments in both cell lines, but only the CCE.33 DNA contains 8.9-kb *BamHI* and 6.2-kb *EcoRI* fragments predicted for successful targeting. These characteristic CCE.33 fragments also hybridize to the *neo* probe, which in addition detects a predicted 11.3 *EcoRI* fragment. The *neo*-hybridizing fragments in CCE.24 DNA resulted from a random integration of the replacement vector. The size markers (lanes M), *HindIII* fragment of phage λ DNA.

To screen for germ-line transmission, male MF1 and C57Bl/6 chimaeras were mated to appropriate tester females, and the litters scored for progeny with CCE.33-derived agouti pigmentation (Table 2b). Three of the seven chimaeric MF1 males that we tested produced agouti progeny, albeit with low frequency. Similarly, four of seven chimaeric C57Bl/6 males produced agouti progeny, but the frequency of transmission was sig-



FIG. 2 Example of 9-day-old wild-type and heterozygous littermates (progeny of animal 'A'; Table 2b).

nificantly higher. Notably, two males (animals 'A' and 'B'; Table 2b) have produced exclusively agouti progeny, apparently because their germ lines were derived entirely from the injected CCE.33 cells.

Unexpectedly, we observed that among the agouti progeny sired by males 'A', 'B' and 'C' (a total of 77 animals from 11 litters) there were animals with considerably smaller body size than their littermates (Fig. 2). Southern-blot analysis of tail DNA from 33 of the progeny of these three males showed that all the normal-size pups were homozygous for the wild-type IGF-II gene (22 samples), whereas the smaller pups were all heterozygous and possessed a disrupted IGF-II allele (11 samples). Of the surviving progeny, 27 are small, and 40 have normal size. We could recover only four of the ten pups that died at or soon after birth. These pups had a small body size and, as genotyping confirmed, were heterozygotes. Assuming that all of the animals that did not survive were small, ~50% of all progeny (37 out of 77) were heterozygous, and 50% wild-type (40 out of 77), as expected.

The postnatal growth curves of wild-type and heterozygous animals (Fig. 3) showed that the rate of weight increase, as a function of the weight at birth, was the same in the two groups. The ratio of heterozygous to normal body weight was practically constant (~0.6) throughout postnatal development, up to at least day 30. A similar weight ratio was observed when two litters of embryos were examined at days 16–19 post coitum. All of these embryos (21) were genotyped (eight normal-size embryos were wild-type, and 13 smaller embryos were heterozygous). Therefore, the mutation seems to exert its effect at a stage earlier than embryonic day 16.

Because the weight of the heterozygotes was about half that of the wild-type animals, we initially surmised a gene dosage effect. But preliminary analysis of the IGF-II RNA level in embryonic stages indicates a more complex situation. Parallel slot-blot assays of RNA from genotyped embryos of two litters, using an exon E2-specific probe and a probe for an RNA standard, revealed that the amount of IGF-II transcripts derived from the intact allele in heterozygotes was about 10-fold less than in wild-type embryos (data not shown). We have not yet examined the relative concentrations of the IGF-II polypeptide.

Our results demonstrate a causal relationship between the elimination of the function of an IGF-II allele and a growth-deficiency phenotype that becomes apparent at least as early as embryonic day 16 and persists after birth. It is unknown whether the mutation affects the embryo directly or indirectly (or both). *In situ* hybridization analysis of the mouse conceptus has demonstrated that all of the components that eventually form the chorioallantoic placenta express the IGF-II gene (J. Lee, J. Pintar, and A. E., manuscript in preparation). The possibility exists that the growth-deficiency phenotype is the indirect consequence of impaired placental trophic functions. In this regard, we note that in the litter of 16-day-old embryos examined, the average weight ratio of heterozygotes to wild-type embryos was the same as that of their respective placentas.

Apart from their small size, the heterozygous animals appear normal and have reached sexual maturity; both male and female

(a) Frequency of generation of chimaeras by blastocyst injection of CCE.33 cells*				
Genotype of host blastocyst	Blastocysts transferred	Progeny born (% of blastocysts)	Chimaeras (% of progeny born)	Phenotypic males (% of chimaeras)
CD-1	64	24 (38)	6 (25)	ND
MF1	58	24 (41)	13 (54)	7 (54)
C57Bl/6	135	61 (45)	22 (36)	15 (68)

(b) Breeding data from male chimaeras		
Genotype of host blastocyst	Male chimaera	Ratio of ES cell-derived progeny to progeny sired
MF-1	1	1/22
	2	1/33
	3	1/35
	4	0/49
	5	0/12
	6	0/25
	7	0/32
C57Bl/6	A	64/64
	B	11/11
	C	2/6
	D	3/9
	E	0/22
	F	0/14
	G	0/8

* Ten to fifteen cells at passage 15 were injected per blastocyst. ND, not determined.

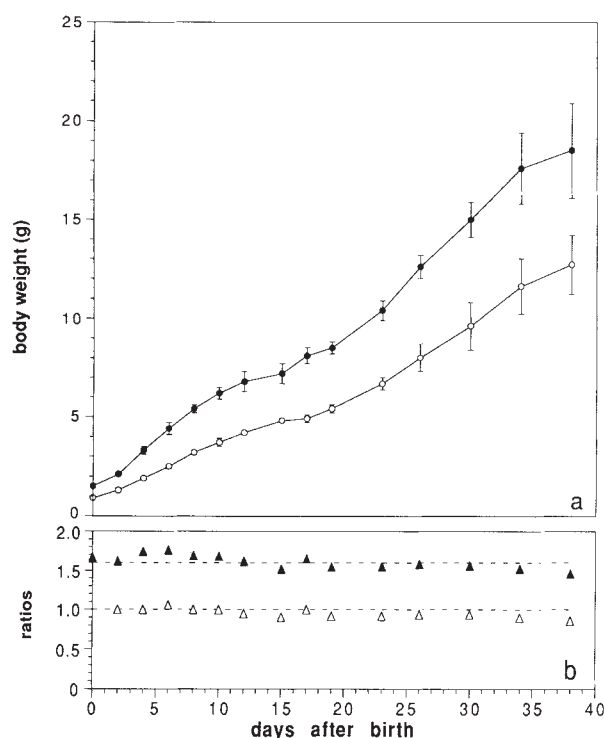


FIG. 3 *a*, Growth curves of wild-type (●) and heterozygous (○) littermates (progeny of animal 'A'; Table 2*b*). The mean weights of seven wild-type and three heterozygous animals of both sexes are plotted (a sex difference in weight was not observed). The width of the vertical bars represents 2 s.d. from the mean. *b*, Ratio of the wild-type to heterozygote mean weight at each time point is indicated (▲). The average of all ratios (dotted line) was 1.6 ± 0.1 . The corresponding values in smaller litters (two from animal 'A' and one from animal 'C') were 1.7 ± 0.2 , 1.8 ± 0.08 and 1.9 ± 0.06 (wild-type to heterozygous animals: 4/3, 5/1 and 1/1 respectively). From the data in A, we also calculated the percentage increase (*I*) of mean weight at each time point (W_t) as compared with the weight at birth (W_0), that is: $I = (W_t - W_0) \times 100 / W_0$. The ratios $I_{\text{wild-type}} / I_{\text{heterozygote}}$ at the various time points (Δ) did not deviate significantly from 1.0 (dotted line) for at least 30 days.

heterozygotes are fertile and transmit the mutation after mating with wild-type animals. In five litters of F2 progeny from two male heterozygotes, 22 animals were normal and 21 animals showed the growth-deficiency phenotype. To examine further the biological role of IGF-II, we are continuing the molecular analysis of the heterozygotes and have commenced inter-crosses to study the consequences of the mutation in the homozygous state. □

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Mediation of meiotic and early mitotic chromosome segregation in *Drosophila* by a protein related to kinesin

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CONTRARY to the traditional view that microtubules pull chromosomes polewards during the anaphase stage of meiotic and mitotic cell divisions, new evidence suggests that the chromosome movements are driven by a motor located at the kinetochore¹⁻³. The process of chromosome segregation involves proper arrangement of kinetochores for spindle attachment, followed by spindle attachment and chromosome movement. Mechanisms in *Drosophila* for chromosome segregation in meiosis differ in males and females⁴, implying the action of different gene products in the two sexes. A product encoded at the *claret* locus in *Drosophila* is required for normal chromosome segregation in meiosis in females and in early mitotic divisions of the embryo. Here we show that the predicted amino-acid sequence of this product is related to the heavy chain of kinesin⁵. The conserved region corresponds to the kinesin motor domain and includes the ATP-binding site and a region that can bind microtubules. A second region contains a leucine repeat motif which may mediate protein-subunit interactions necessary for attachment of chromosomes to the spindle. The mutant phenotype of chromosome nondisjunction and loss, and its similarity to the kinesin ATP-binding domain, suggest that the product encoded at *claret* not only stabilizes chromosome attachments to the spindle, but may also be a motor that drives chromosome segregation in female meiosis.

The *claret* locus in *Drosophila* encodes two products, one needed for wild-type eye colour and one for normal chromosome segregation. The genes encoding these products are separate and are divergently transcribed⁷. Females carrying mutations in the segregational gene at the *claret* locus show high-frequency meiotic nondisjunction and loss affecting all chromosomes, although recombination between homologues is normal⁸. Nondisjunctional products and products of chromosome loss can be recovered as abnormal offspring that carry two maternal homologues instead of one, or as offspring that have lost the maternally contributed chromosome. Embryos produced by mutant females also show high-frequency loss of chromosomes in early mitotic divisions. Because loss of the X or fourth chromosome is nonlethal in *Drosophila*, offspring that have undergone loss of these chromosomes in early mitosis can be recovered as gynandromorphs (X/X, X/O mosaics) or as haplo-4, diplo-4 mosaics. This early embryonic chromosome loss is a maternal effect that almost exclusively affects maternal chromosomes. The basis of this effect is likely to be a maternal product that is transmitted with maternal chromosomes (D. J. Comma, A. S. Horne and S.A.H., manuscript in preparation).

The *claret* locus was cloned by chromosome walking and identified by a P element insertion in a hybrid dysgenesis-induced mutant⁷ (Fig. 1). Germline transformation of the 11.5-kilobase (kb) *XhoI* fragment (Fig. 1) rescued the nondisjunctional phenotype of *claret* mutant alleles⁷. Transformation experiments now show that the 5.3-kb *EcoRI*-*XhoI* fragment (Fig. 1) is sufficient to rescue the effect of *claret* mutant alleles

on meiotic and mitotic chromosome segregation (Table 1). This rescue experiment clearly implicates the 2.2-kb RNA, which is completely encoded by the 5.3-kb fragment, as the transcript of the segregational gene at *claret*. The 2.2-kb RNA is present in females, where it is found predominantly in the ovary⁷. It is absent from RNA of *claret*nd (*ca*nd) and *claret*^{Cm} (*ca*^{Cm}) females; *ca*nd and *ca*^{Cm} are therefore null alleles for the product of chromosome segregation.

Several full-length or nearly full-length complementary DNAs that correspond to the 2.2-kb transcript were recovered from an ovary cDNA library⁷. Both strands of one of these cDNAs (ϕ 8e2) were completely sequenced, together with both strands of a shorter, 1-kb cDNA (ϕ 8e1). Figure 2 shows the sequence obtained for the two cDNAs. The 1-kb cDNA is shorter at both the 5' and 3' ends, but otherwise is identical in sequence to the 2.1-kb cDNA. The 1-kb cDNA contains a run of A residues 23 base pairs (bp) after the consensus polyadenylation signal⁹, AATAAA, which is underlined. The 2.1-kb cDNA contains genomic *EcoRI* sites at both its 3' and 5' ends. The corresponding messenger RNA is presumed to have a poly(A) tail that lies just to the right of the 3' *EcoRI* site, because two polyadenylation signals of AATAAA begin 26 bp and 18 bp, respectively, upstream of the end of the *EcoRI* site. The protein sequence is shown as beginning with the first in-frame ATG, although this ATG is not in the sequence context that has been shown to flank most *Drosophila* translational start sites¹⁰. The reasons for the deviation from the consensus sequence are not certain, although one possibility is that the 5' end of the gene extends beyond the region shown. But the 2,311-bp length of the region and the approximate 2.2-kb size of the *ca*nd mRNA⁷ indicate that the mRNA is likely to be completely encoded by the region indicated. A second possibility is that the ATG translational start for this transcript is atypical with respect to its sequence context. Beginning with this ATG, an open reading frame of 700 amino acids is predicted. The termination codon at position 2,199 is TAA.

A search for sequence similarity was carried out as described previously¹¹ using the Genbank 61 and EMBL 20 databases. Each nucleotide sequence database entry was translated in its six possible reading frames and compared with the open reading frame from the *claret* segregational product. A striking similarity was found to *Drosophila* kinesin heavy chain⁵ (Fig. 3).

Kinesin induces movement of microtubules, or vesicles on microtubules, upon hydrolysis of ATP¹². The gene encoding the *Drosophila* kinesin heavy chain has been cloned¹³ and sequenced⁵. The amino-acid sequence predicted from the nucleotide sequence suggests that the kinesin heavy chain comprises three domains: a globular N-terminal motor domain, a stalk region capable of forming α -helical coiled coil and a C-terminal domain that may interact with other proteins or cellular

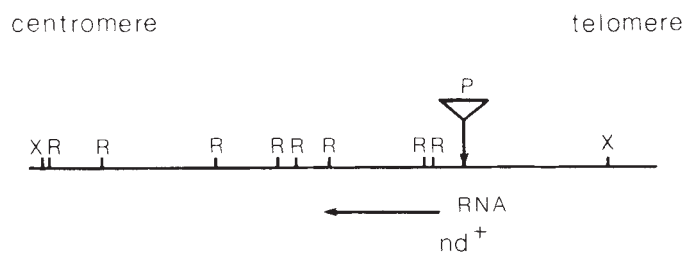


FIG. 1 The *claret* locus in *D. melanogaster*. Restriction sites for *XhoI* (X) and *EcoRI* (R) are shown. The site of P-element insertion in a hybrid dysgenesis-induced mutant (*ca*^{P6}; ref. 7), and the location and direction of transcription of the mRNA for the segregational gene are indicated. The 5.3-kb *EcoRI*-*XhoI* fragment that rescues the mutant segregational defect is shown at the bottom. The 0.2-kb *EcoRI* fragment adjacent to the P-element insertion site was not deleted in previous studies.

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Tests for X and fourth chromosome segregation were carried out by mating females of the indicated genotypes to y^2w^{Bf}/B^SY males that carry a recessively marked X and a dominantly marked Y chromosome. Nondisjunction in female meiosis results in X, X and nullo-X gametes that are recovered as X/X/ B^SY (B^S) females and X/O males that carry paternal X chromosomes (y w males). Meiotic loss of X chromosomes in females also results in nullo-X gametes that are recovered as X/O (y w males). Nondisjunctional X/X/ B^SY females and X/O males are expected to be recovered in equal numbers because they represent reciprocal products of the same event. The excess of X/O males is attributed to chromosome loss in meiosis²³. Haplo-4 females or males exhibit a Minute (M) phenotype and are of variable viability. Gynandromorphs (gyn) and Minute mosaics arise as a consequence of X or fourth chromosome loss in early mitotic divisions of the embryo. G418^R indicates the presence of the 5.3-kb *EcoRI*-*XhoI* transgene in the transformant. Females heterozygous for ca^{nd} and ca^{cm} produce high frequencies (29.5%; line 1) of abnormal offspring due to chromosome loss and meiotic nondisjunction of chromosomes. The transgene, indicated by G418^R (line 2), completely rescues the mutant effect on chromosome segregation. The frequency of abnormal offspring produced by mutant females carrying the transgene is not significantly different from that produced by ca/ca^{cm} females (line 3), in which the recessive segregational defect of ca^{cm} is complemented by the ca chromosome (which is mutant for the eye-colour gene at the *claret* locus, but not the segregational gene). Although only segregation of the X and fourth chromosomes was monitored in this experiment, ca^{nd} causes nondisjunction and loss of all chromosomes⁸.

The N-terminal region of the ca^{nd+} product contains a heptapeptide leucine repeat (Fig. 2) which, in an α -helix, is characteristic of coiled-coil interactions. The leucine repeat is disrupted by glutamine in position d of the third repeat. Glutamine has been found in a similar position in tropomyosin¹⁶, and is therefore not inconsistent with coiled-coil interactions. There is a proline in position a of the fifth repeat. Proline in α -helices is uncommon. However, it does occur, and in these instances it results in a bent helix¹⁷. This may be of functional importance. The leucine repeat region in the ca^{nd+} product may thus mediate protein subunit interactions in the manner proposed for other leucine repeat-containing proteins¹⁸.

FIG. 2 Nucleotide sequence of *claret* segregational product cDNA. cDNAs ϕ 8e2 and ϕ 8e1 were sequenced using the dideoxy chain termination method. The first 175 nucleotides are from an overlapping genomic DNA clone. Shown is the sense strand together with the predicted amino-acid sequence. The ATG stop codon is marked with an asterisk. The region of heptamer leucine repeats is boxed. The reading frame was confirmed by expressing the C-terminal *Bam*HI-*Eco*RI fragment from the longer of the two cDNAs in the pET-5 a, b and c vectors²⁴ in the three possible reading frames. After induction with IPTG, the T7 gene-10 fusion protein was detected on a western blot by reaction with an antibody directed against the gene-10 portion of the fusion protein. The relative molecular mass of the fusion protein produced by the insertion cloned in pET-5 b is estimated to be ~24,000 (24K) compared with the expected *M_r* of 20,849 predicted from translation of the DNA sequence. The fusion proteins produced by pET-5 a and c have predicted *M_r*s 5,234 and 2,668, respectively. Proteins corresponding to these products were not detected on the blots, presumably because the products were run off the end of the gel. This experiment indicates that the correct reading frame for the cDNA is the b frame (in frame with the GAT of the *Bam*HI site) which corresponds to the long open reading frame present in the cDNA. This sequence has been submitted to Genbank.

[illegible]

ca	E L H N T V M D L R G N I R V F C R I R P P L E S E E N R M C C T W T Y H - - D E S T V E L Q S I D A Q A K S K M G Q Q I E S F D Q V F	402
kin	M S A E R E I P A E D S I K V V C R F R P L N D S E E K A G - S K F V V K - F P - N N - V E E N C I S - I A - G K - - - - V Y L F D K V F	59
KAR	T L H N E L O F L R G N I R V V C R I R P A L K N L E N S D T S L I N V N E F D D N S G V Q - S M E V T K I Q N T A Q V H E F K F D K I F	442
bim	I E R E I N E D T S I H V V V R C R G R N E R E V K E N S G V V L Q T E - - G V K G K T V E L S M G P N A V S N K T Y T - - - - F D K V F	134
ATP-binding		
ca	H P L S S Q S D I F - E M V S P L I Q S A L D G Y N I C I F A Y G Q T G S G K T Y T M D G V P - E S V - - - - - G V I P R T V D L L F	462
kin	K P N A S Q E K V Y N E A A K S I V T D V L A G Y N G I F A Y G Q T S S G K T H T M E G V I G D S V - - - - - K O G I I P R I V N D I F	123
KAR	D Q D T N V D V F K E V I - G Q L V Q S L D G Y N V C I F A Y G Q T G S G K T P T M L - N P G D - - - - - P S T I S H I F	500
bim	S A A A D Q I T V Y E D V L V L P I V T E M L A G Y N C T I F A Y G Q T G T G K T Y T M S G D M T D T L G I L S D N A G I I P R V L Y S L F	203
ca	D S I R G Y - R N L G W E Y E I K A T F L E I Y N E V L Y D L L S N E Q K D M E I R M A - - K N N K N D I Y V S N I T E E T V L D P N H L	528
kin	N H I - - Y A M E V N L E F H I K V S Y Y E I Y M D K I R D L L D V S K V N L S V H E D - - K N R V P Y V K - - G A T E R F V S S P E D V	186
KAR	N W I N K L K T K - G W D Y K V N C E F I E I Y N E N I V D L L R S D N N N K E D T S I G L K H E I R H D Q E T K T T I T N V T S C K L	568
bim	A K L A D T E S T V K C S - - - - - F I E L Y N E E L R D L L S A E E N P K L K I Y D N E Q K K G H M S T L V Q G M E E T Y I D S A T A	266
ca	R H - L M H T - - - - - A K M N R A T A S T A G C N E R S S R S H A V T K L E L - I G R H - - A E K Q E I S V G S I N L V D L A G S E S P -	587
kin	F E - V I E - - - - - G K S N R H I A V T A S N E H S S R S H S V F L I N V - K O E N - - L E N Q K K L S G L V L V D L A G S E K V S	246
KAR	E S E E M V E I I L K K A N K L R S T A S T A S N E H S S R S H S V F I H L - S G S N - - A K T G A H S Y G T L N L V D L A G S E R I N	634
bim	G I - K L L Q - - - - - Q G S H K R Q V A T K C N D L S S R S H T V F T I T V N I R T T E S G E E Y V C P G K L N L V D L A G S E N I G	330
microtubule-binding		
ca	K T S - - - T R M T E T K N I N R S L S E L T N V I A L L Q - - K - Q D H I P Y R N S K L T H L L M P S L G G N S K T L M F I N V S P F	650
kin	K T G A E G T V L D E A K N I N K S L S A L G N V I S A L A D - - G N K T H I P Y R D S K L T R I L Q E S L G G N A R I T I V I C S P A	313
KAR	V S Q V V G D R L R E T Q W I N K S L S C L G D V I H A L G Q P D S T K R H I P Y R N S K L T Y L L Q Y S L T G D S K T L M F V N I S P S	703
bim	R S G A E N K R A T E A G L I N K S L L T L G R V I N A L V - - D K S - Q H I P Y R E S K L T R L L Q D S L G G R T K T C I I A T M S P A	396
ca	Q D C F Q E S V K S L R F A S V N S C K M T K A K R	677
kin	S F N E S E T K S T L D F G R R A K T V K N V C V N	340
KAR	S S H I N E T L N S L R F A S K V N S T R L V S R K *	729
bim	R S N L E E T I S T L D V A F R A K N I R N K P Q I N	423

FIG. 3 Comparison of the predicted amino-acid sequences of the *claret* segregational product and *Drosophila* kinesin heavy chain. Predicted amino-acid sequences from KAR3 (ref. 20) and bimC (ref. 21) are included in the comparison. Identical residues are boxed. The putative ATP-binding site is indicated. The underlined sequences correspond to a region that can bind

microtubules⁵. The N-terminal boundary of this region lies between or distal to kinesin residues 129 and 174. The uncertainty in position of this boundary (indicated by a single line) reflects the uncertainty in translation initiation site of the 5' deletion constructs that were used in the mapping experiments⁵. The C-terminal boundary is at, or proximal to, kinesin residue 331.

loss, the wild-type product is predicted to be involved in chromosome distribution, perhaps by affecting kinetochore position or attachment of the chromosomes to the spindle. Our findings indicate that a protein necessary for proper chromosome segregation in meiosis has features of a microtubule-binding motor molecule. This molecule could mediate binding of chromosomes to spindle microtubules and generate the force needed for movement of chromosomes during meiosis. Such chromosome movement occurs during prometaphase where it may be needed for proper positioning of chromosomes for spindle attachment, as well as during anaphase. An alternative role is in spindle elongation during the later stage of anaphase.

Spindle microtubules are orientated with their minus ends at the spindle pole and their plus or fast-growing ends pointed away from the pole. Spindle microtubule polarity thus requires a minus-end-directed motor for movement of chromosomes along the kinetochore microtubules to the poles. Kinesin *in vitro* shows only plus-end-directed movement. It is possible that the *ca*^{nd+} product is minus-end directed, as required for a chromosome-associated anaphase motor. Its similarity to kinesin, however, suggests that the *claret* segregational product is a plus-end-directed motor. Such a plus-end-directed, ATP-dependent activity has been detected previously¹⁹ and proposed to be involved in prometaphase chromosome movements.

The broad distribution of kinesin has led to the prediction that it or related products will be involved in many microtubule-based, cellular motile processes. Several kinesin-related molecules have recently been discovered^{20,21}. The *ca*^{nd+} product is among these newly discovered kinesin-related molecules, and is clearly implicated in chromosome segregation. There are probably several molecules in *Drosophila* that perform functions similar to that of the *claret* segregational protein: the *ca*^{nd+} product is active predominantly in females and in meiosis I; a homologous male-specific meiotic product is likely to exist and may be encoded by the *paternal loss* (*pal*) locus²² and products that act in meiosis II and in mitosis (D. J. Comma, A. S. Horne and S.A.H., manuscript in preparation) are also predicted. We

propose the term 'disjunctin' to denote members of this predicted family of related products involved in disjunction of chromosomes in meiosis and mitosis.

Note added in proof: After we discovered that *ca*^{nd+} was kinesin-related, we sent our DNA sequence to L. Goldstein who found it matched his partial sequence of a previously unidentified PCR clone obtained by homology to the kinesin motor domain. □

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Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients

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KAPOSI'S sarcoma (KS) is frequently associated with human immunodeficiency virus-1 (HIV-1) infection¹. Supernatants from HIV-1-infected T cells carrying the CD4 antigen promote the growth of cells derived from KS lesions of AIDS patients (AIDS-KS cells)², and the HIV-1 *tat* gene, introduced into the germ line of mice, induces skin lesions closely resembling KS³. Here we report that the *tat* gene product (Tat) is released from both HIV-1-acutely infected H9 cells and *tat*-transfected COS-1 cells. These Tat-containing supernatants specifically promote growth of AIDS-KS cells which are inhibited by anti-Tat antibodies; recombinant Tat has the same growth-promoting properties. Therefore a viral regulatory gene product can be released as a biologically active protein and directly act as a growth stimulator. These and previous data³ indicate that extracellular Tat could be involved in the development or progression, or both, of KS in HIV-1-infected individuals.

We recently developed a system for the long-term culture of spindle-like cells derived from KS lesions of AIDS patients (AIDS-KS cells)² dependent on conditioned media (CM) derived from activated T cells carrying the CD4 antigen (ref. 2, and B. Ensoli *et al.*, manuscript in preparation) or HIV-1-infected cells². Morphological and immunohistochemical studies indicated that AIDS-KS cells are of mesenchymal cell origin and share features with endothelial and smooth muscle

cells (ref. 4; and H. A. Welch, S. Nakamura, S.Z.S., R.C.G. and J. Folkman, manuscript submitted), both potential progenitors of the suspected tumour cells (spindle cells) of KS^{4,5,7}. Transplantation of AIDS-KS cells into nude mice produced mouse lesions closely resembling KS⁴, probably by release of specific cytokines with effects relevant to the histogenesis of KS lesions, including neoangiogenesis (refs 4, 8, 9; and E. W. Thompson *et al.*, manuscript submitted). The presence of KS-growth promoting activity in CM from HIV-infected CD4⁺ T cells, the absence of HIV-1 sequences in DNA from KS tissue or cultured cells^{4,6} and the observation that transgenic mice carrying the *tat* gene develop KS-like lesions and express *tat* in the skin but not in the tumour cells³, indicate that the role of HIV-1 in KS is indirect, and that Tat itself might be released by infected cells and promote activation and growth of target cells involved in the formation of KS.

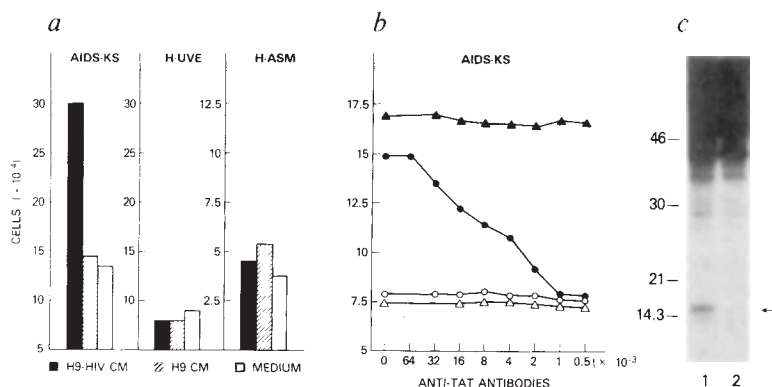
To test this hypothesis, we examined the effect on growth of CM from HIV-1-infected T cells (H9)¹⁰ on AIDS-KS cells (AIDS-KS3 cell clone)^{2,4,8}, and on normal control cells (human umbilical vein endothelial cells, H-UVE; and human newborn aortic smooth muscle cells, H-ASM). H9-HIV-1-derived CM, but not H9 CM, specifically stimulated AIDS-KS cell proliferation but had no effect on H-UVE or H-ASM growth after 6 days of culture (Fig. 1a), or in prolonged assays (2 weeks) (data not shown). The average stimulation of AIDS-KS cell growth (5–6 days assay) was 2.0-fold (1.7- to 3.3-fold) for HIV-1 CM as compared to 2.5-fold (1.9- to 3.6-fold) with the positive control (CM from human T-lymphotrophic virus-II (HTLV-II)-transformed CD4 T cells)². The variability observed in different experiments was related to cell passage number and the level of basal cell growth.

The proliferation of AIDS-KS cells stimulated by H9-HIV-1 CM was specifically inhibited by anti-Tat antibodies, whereas there was no reduction of cell growth for control media or positive control (Fig. 1b). This indicates that Tat is responsible for the growth effect present in CM from HIV-1-infected H9 cells, implying that Tat is released into the extracellular fluid.

Using radioimmunoprecipitation analysis (RIPA) with affinity-purified anti-Tat antibodies, we detected a distinct band of the same apparent relative molecular mass (M_r) as intracellular Tat (M_r 15,000; 15 K)¹¹ in CM from H9-HIV-1-acutely infected cells (Fig. 1c). Extracellular Tat was detectable 8–16 days after cell-free infection of H9 cells, and its presence corre-

FIG. 1 Tat is involved in AIDS-KS cell response to HIV-infected T cells. **a**, Growth response of AIDS-KS and control cells to CM from HIV-1-infected or uninfected H9 cells. CM were diluted 1:4. **b**, Specific block of AIDS-KS cell growth response to H9-HIV-1-derived CM by anti-Tat antibodies. ●, H9-HIV-1-derived CM (1:16); ○, H9-derived CM (1:16); △, medium without CM; ▲, HTLV-II-derived CM (1:16). **c**, RIPA of Tat in CM from HIV-1-infected or uninfected H9 cells. Lanes 1 and 2, CM from HIV-1-infected or uninfected H9 cells, respectively. Arrow, Tat in CM from infected cells.

METHODS. CM from HIV-1-infected (IIIB isolate) or uninfected H9 cells ($1 \times 10^6 \text{ ml}^{-1}$) were collected from day 4–24 after cell-free infection, centrifuged at low (2,000g) and high speed (30,000 r.p.m.) for 1 h, stored at -20°C and individually tested. Effects of CM 10 days after infection are shown. Cells were seeded in 25-cm² flasks (AIDS-KS, 5×10^4 ; H-UVE, 1×10^5) or in 12-well plates (H-ASM, 5×10^3 per well). Medium specimens were replaced on day 3. The cell number was determined after trypsinization of cells on day 5 or 6 of culture by both Trypan blue staining and Coulter-counter measurements. For AIDS-KS cells, CM from HTLV-II-transformed T cells (HTLV-II CM, dilution 1:4), and for H-UVE cells, ECGS ($30 \mu\text{g ml}^{-1}$) and heparin ($45 \mu\text{g ml}^{-1}$), were used as positive controls², with values of 35.2×10^4 and 38×10^4 , respectively. Before each assay, AIDS-KS cells were maintained for 4–15 days in medium without HTLV-II CM and incubated in serum-free media for 4 h. Experiments were repeated twice with the same CM and several other times with different CM preparations. Similar results were also obtained on other AIDS-KS cell cultures (AIDS-KS1 and AIDS-KS2)^{2,4}. **b**, AIDS-KS3 cells were cultured and plated as in **a**. CM or medium alone were preincubated (7 h to overnight) with or without serial dilutions of rabbit anti-Tat polyclonal antibodies ($\sim 7 \mu\text{g } \mu\text{l}^{-1}$) obtained by immunization with recombinant purified Tat expressed in *E. coli* and purified by affinity chromatography using purified Tat. Fresh media treated identically were replaced on day 3. The cell number



was determined on day 5 or 6 of culture, as described. The experiments were reproduced with a Tat antiserum from the same source and with two different anti-Tat rabbit sera (obtained by immunization with a Tat synthetic peptide (residues 1–61 of N terminus). The effects of CM 12 days after infection are shown. Results were reproduced with other CM preparations (all between 10 to 15 days after infection). **c**, On day 12 post-infection, cells ($1 \times 10^6 \text{ ml}^{-1}$) were metabolically labelled with [³⁵S]methionine and [³⁵S]cysteine ($150 \mu\text{Ci ml}^{-1}$ each) for 24 h, supernatants were centrifuged (40,000 r.p.m., 1 h, 4°C) and precleared for 5 h with normal rabbit serum and protein A-Sepharose (Boehringer). Supernatants (4 ml) were incubated overnight with anti-Tat affinity-purified antibodies (dilution, 1:100) and proteins separated by 15% SDS-PAGE²⁰ (3-day exposure). M_r markers (Rainbow, Amersham) are indicated ($M_r \times 10^{-3}$).

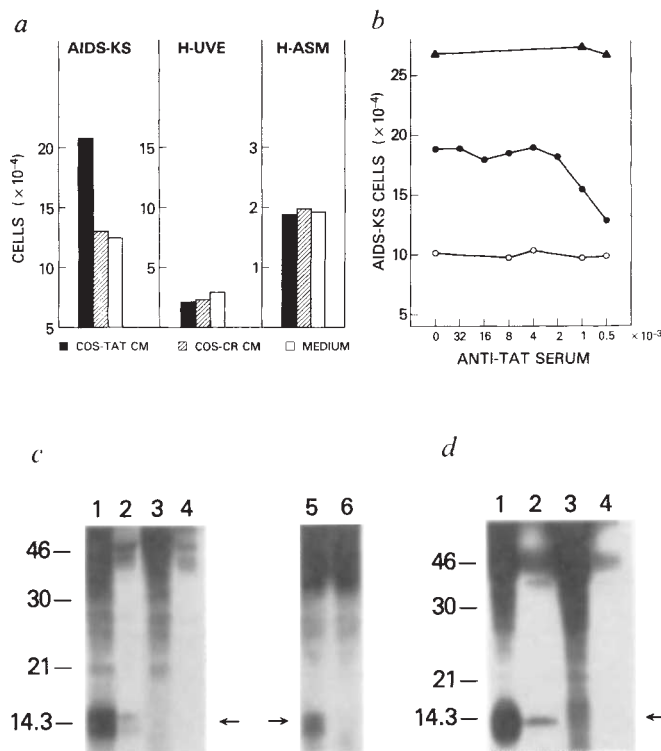
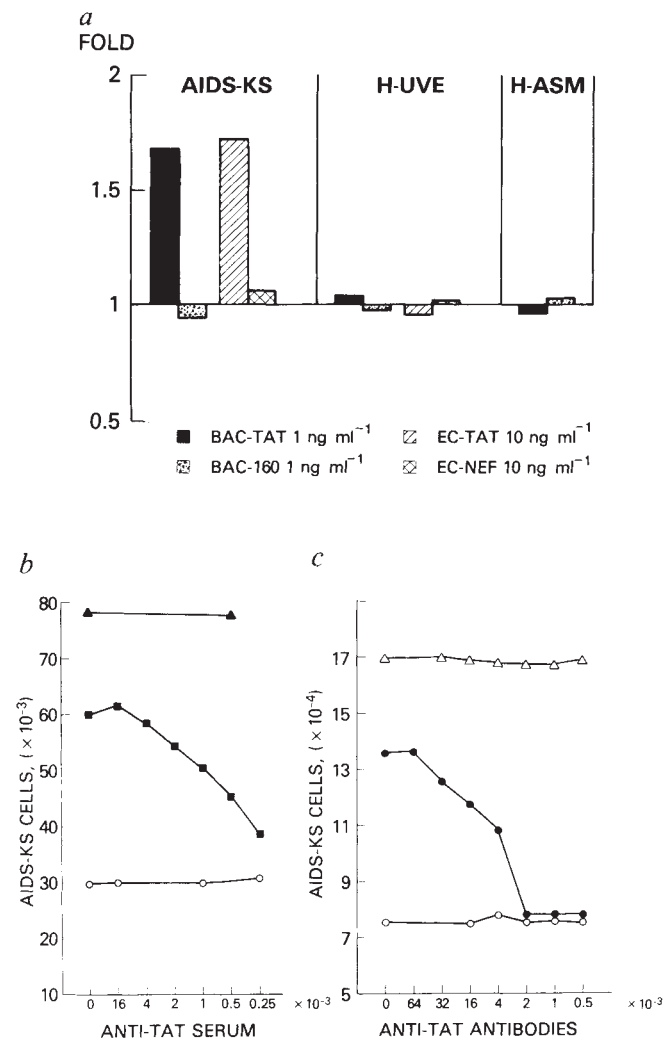


FIG. 2 Role of Tat in growth response of AIDS-KS cells to Tat-transfected COS cells. **a**, Growth response of AIDS-KS cells, H-UVe and H-ASM cells to CM from COS-1 cells transfected with a *tat*-expressing vector (COS-TAT). CM diluted 1:16. **b**, Specific block of AIDS-KS cell growth response to COS-TAT-derived CM by anti-Tat antiserum. ●, COS-TAT-derived CM (1:16); ○, media alone without CM; ▲, HTLV-II-derived CM (1:16). **c**, RIPA of Tat present in cell extract and supernatant from COS-TAT transfected and metabolically labelled cells, and competition assay of extracellular Tat. Lanes 1 and 2, cell extract and supernatant from COS-TAT cells; lanes 3 and 4, cell extract and supernatant from COS-CR cells, respectively, precipitated with anti-Tat antiserum; lanes 5 and 6, supernatants from COS-TAT cells precipitated with anti-Tat antiserum, before (lane 5) and after (lane 6) preincubation of the antiserum with recombinant purified Tat. The arrows indicate the Tat products. **d**, RIPA of Tat in COS-TAT-transfected cells. Lanes 1 and 3, cell extracts; lanes 2 and 4, supernatants from COS-TAT- and COS-CR-transfected cells, respectively, precipitated with affinity-purified anti-Tat antibodies. **METHODS.** **a**, Cells were plated (AIDS-KS3, 5×10^4 ; H-UVe cells, 5×10^4 per 25 cm² flask; H-ASM cells, 2.5×10^3 per well) and cell number determined on day 5 (AIDS-KS), day 6 (H-ASM) or day 9 (H-UVe). In the experiment shown, the positive controls gave values of 28.5×10^4 for AIDS-KS cells (HTLV-II CM) and 20.8×10^4 for H-UVe cells (ECGS and heparin). COS-1 cells ($5-7 \times 10^6$ cells) were transfected by electroporation²¹ with a plasmid expressing *tat* (pcv-tat) or a control plasmid DNA (30 µg each). Ten hours after transfection, cells were split (1:2), washed twice in PBS and incubated (46 h) in RPMI 1640 medium supplemented with 5% dialysed FCS and 1% Nutridoma-HU (Boehringer). CM were collected, centrifuged and stored as described in Fig. 1a. Growth assays were repeated once or twice for each transfection experiment (three). The experiments were also reproduced with AIDS-KS1 cells. **b**, Blocking experiment was performed on AIDS-KS3 cells as described in Fig. 1b and cells counted on day 6. The antiserum used was raised against a Tat synthetic peptide. The experiment was repeated twice and with a different Tat serum. **c**, **d**, COS-1 cells were transfected as described in **a**. Cells were metabolically labelled 56 h post-transfection for 6–8 h, as described in Fig. 1c. Cell extracts (0.5 ml) and CM (1.5 ml for lanes 1 to 4 and 4 ml for lanes 5 and 6) were precleared and incubated overnight with anti-Tat serum (1:100) used for the blocking experiments shown. For the competition assay, the anti-Tat serum (1:100) was preincubated (2 h) with 6 µg recombinant purified Tat (expressed in *E. coli*). Exposure was overnight. The experiments were reproduced with all the different anti-Tat sera available. *M_r* markers are indicated to the left (*M_r* × 10⁻³).

FIG. 3 Specific growth response of AIDS-KS3 cells to recombinant Tat. **a**, Growth response of AIDS-KS3 and control cells to recombinant Tat proteins or to recombinant control proteins (gp160 and Nef). BAC-TAT and BAC-160, baculovirus-derived Tat and gp160 proteins at 1 ng ml⁻¹. EC-TAT and EC-NEF, *E. coli*-derived Tat and Nef proteins at 10 ng ml⁻¹. **b**, **c**, Block of AIDS-KS cell growth response to EC-TAT (**b**) or BAC-TAT proteins (**c**) by anti-Tat antibodies. ■, EC-TAT at the final concentration of 10 ng ml⁻¹; ●, BAC-TAT at the final concentration of 1 ng ml⁻¹; ○, media alone without CM; ▲, △, HTLV-II-derived CM (1:16).

METHODS. **a**, BAC-TAT and BAC-160 were expressed in the baculovirus system (Repligen) (~5% of total protein content); EC-TAT and EC-NEF were expressed in the *E. coli* system (Repligen) (~10% of total protein content). DTT (5 mM) was added to the stock protein preparations to prevent oxidation and equivalent dilutions of the same compound were also added to the positive and negative controls. Proteins and media were replaced on day 3. The cell number was determined on day 6 and expressed as fold increase compared with the values of basal cell growth (media alone, one fold). The positive controls gave a twofold increase for AIDS-KS3 cells (HTLV-II CM) and 2.05-fold for H-UVe cells (ECGS and heparin), respectively. Results shown are the average of experiments repeated five times at the same conditions for AIDS-KS3 cells (s.d. values <5%) and twice for H-UVe cells and H-ASM cells. A similar growth response was also obtained with AIDS-KS1 cells, or after 21 days of assay for all cell types. Activity of both recombinant Tat proteins (10 µg) was tested on HeLa cells carrying the HIV-1 LTR-linked chloramphenicol acetyltransferase (CAT) gene or by transient transfections and quantified by CAT assays as previously shown²⁰. The recombinant proteins were also tested by western blot analysis. The range of activity in inducing AIDS-KS cell proliferation was 0.5–10 ng per ml final total protein concentration for BAC-TAT with a peak between 1 and 5 ng ml⁻¹, and 1–20 ng ml⁻¹ for EC-TAT with a peak between 5 and 10 ng ml⁻¹. Some variability in dose response was observed, generally dependent on aging, storage of proteins and cell culture conditions. Loss of activity is probably due to oxidation of Tat¹⁹. All the protein preparations were negative for endotoxin (<0.0001 pg µl⁻¹). **b**, **c**, AIDS-KS3 cells were plated at 1×10^4 per 25-cm² flask (**b**) or at 5×10^4 per 25-cm² flask (**c**). Anti-Tat serum raised against a Tat synthetic peptide was used for EC-TAT, whereas anti-Tat affinity-purified antibodies were preincubated with BAC-TAT. Cell number was determined on day 6. The experiments were repeated twice for each protein preparation. For BAC-TAT, other antibody preparations were also used.



lated with the time of optimal CM growth-promoting effect and with positive blocking effect by anti-Tat antibodies.

To study the proliferative effect induced by Tat and to avoid cell death commonly observed during HIV-1 infection of T cells, we examined the growth-promoting activity in CM of COS-1 cells transfected with *tat*-expressing plasmid (COS-TAT) (Fig. 2a). Specific stimulation of AIDS-KS, but not of H-UVE or H-ASM, cell growth by COS-TAT CM could be reproducibly observed, even in longer assays (2 weeks), and specifically blocked by several anti-Tat sera (Fig. 2b). The same antibodies also detected Tat in both cell extracts and CM of metabolically labelled COS-TAT cells (Fig. 2, c and d). Two protein species ($M_r \sim 14$ K and 16 K) were sometimes detected with several antibodies (Fig. 2c), and both bands were completely competed-out by purified recombinant Tat (Fig. 2c, lane 5). The doublet may correspond to the two forms of Tat expressed from spliced and unspliced messenger RNA¹².

We next tested the growth effects of recombinant Tat expressed in baculovirus (BAC-TAT) and in *Escherichia coli* (EC-TAT) on AIDS-KS cells and control cells. Both BAC-TAT and EC-TAT induced AIDS-KS cell proliferation, but not growth of control cells (for up to 3 weeks). The optimal concentrations were 1 ng ml^{-1} (BAC-TAT) and 10 ng ml^{-1} (EC-TAT) (Fig. 3a). The same concentrations of baculovirus-expressed gp160 and *E. coli*-expressed Nef protein failed to induce AIDS-KS cell growth or growth of control cells. In these experiments there was a 1.7-fold increase of AIDS-KS cell number with Tat, and a 2-fold increase with the positive control. In several other experiments, greater stimulation was observed, and the ability of both Tat preparations to induce AIDS-KS cell proliferation was specifically neutralized by preincubation with several anti-Tat sera (Fig. 3b and c). Because AIDS-KS cells are primary cell cultures of mesenchymal origin and cells of comparable origin (endothelial and smooth muscle cells) normally do not proliferate *in vivo* and need particular conditions to grow *in vitro* (cell culture system and growth factors), the reproducible (about twofold) stimulation of AIDS-KS cells by Tat seems to be significant.

These *in vitro* data show that Tat may provide the primary growth stimulus to AIDS-KS cells, and may have a role *in vivo* in the pathogenesis of HIV-1-associated KS. This does not rule out contribution by other factors at different stages of infection and disease development. Because normal endothelial and smooth muscle cells are not responsive to Tat, the normal KS cell progenitor may require a previous or simultaneous 'priming' event, or both, before becoming Tat-responsive. Alternatively, a different subtype of endothelial or smooth muscle cells, not available at present for *in vitro* study, may be the progenitor of KS spindle cells.

The mechanisms by which Tat is released and promotes growth are unknown. Because cell death is minimal both after infection of H9 cells and in Tat-transfected COS cells, release of Tat is unlikely to be due to cell death. Tat may be a member of the class of proteins that, although lacking a classical signal peptide, are released by cells through an unknown mechanism(s) (interleukin-1 α and -1 β (refs 13, 14), platelet-derived endothelial cell growth factor¹⁵ and basic and acid fibroblast growth factors^{16,17}). Similar to the effects of Tat on AIDS-KS cells, all these cytokines induce their effects on target cells at very low doses (pg-ng).

Exogenous Tat can be taken up by cultured cells, localizes in the nucleus¹⁸ and can depress antigen-mediated T-cell activation response¹⁹, further indicating a role of exogenous Tat in cellular functions. But the amount of Tat necessary for biological effects (μg) is several times that necessary for the growth-promoting effect^{18,19}, indicating that *in vivo* growth stimulation of some mesenchymal target cells by Tat may be more biologically relevant.

Our present results show that a viral regulatory gene product can be released as a biologically active protein and directly act

as a growth stimulator for cells derived from KS lesions of AIDS patients. Although our *in vitro* data do not definitively establish a general mechanism for the *in vivo* genesis of KS, these and previous results³ indicate that, by inducing AIDS-KS cell proliferation, Tat may contribute to the induction or progression, or both, of KS in HIV-1-infected individuals, thus representing one of the links between HIV-1 infection and KS. □

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Ancestral lysozymes reconstructed, neutrality tested, and thermostability linked to hydrocarbon packing

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THE controversy surrounding the idea that neutral mutations dominate protein evolution¹ is attributable in part to the inadequacy of the tools available to evolutionary investigators. With a few exceptions², most investigations into the force driving protein evolution³ have relied on indirect criteria for distinguishing neutral and non-neutral variants. To investigate a particular pathway of molecular evolution, we have reconstructed by site-directed mutagenesis likely ancestral variants of the lysozymes of modern game birds (order Galliformes), tested their activity and thermostability and determined their three-dimensional structure. We focused on amino acids at three positions that are occupied in all known game birds either by the triplet Thr 40, Ile 55, Ser 91, or by the triplet Ser 40, Val 55, Thr 91. We have synthesized proteins representing intermediates along the possible three-step evolutionary pathways between these triplets. Although all of these are active and stable, none of these intermediates is found in known lysozymes. A comparison of the structures and thermostabilities of the variants reveals a linear correlation between the side-chain

volume of the triplet and the thermostability of the protein. Each pathway connecting the two extant triplet sequences includes a variant with a thermostability outside the range of the extant proteins. This observation is consistent with a non-neutral evolutionary pathway. The existence of variants that are more stable than the extant proteins suggests that selection for maximum thermostability may not have been an important factor in the evolution of this enzyme.

In searching for possibly non-neutral pathways of evolution, our attention was drawn to positions 40, 55 and 91 in the amino-acid sequences of game-bird lysozymes. The residues at these positions lie together in the three-dimensional structure (Fig. 1a), just beneath the active-site cleft at the vertex of the putative hinge between the two globular domains^{4,5}. Moreover, amino-acid replacements accumulate nonrandomly at these positions. In the lineages leading from ancestral game birds to American quail and African guinea fowl, the threonine (T), isoleucine (I) and serine (S) residues at positions 40, 55 and 91 are replaced by Ser, Val(V) and Thr, respectively⁶. The probability is low that in these lineages three internally clustered residues would change by chance without any changes occurring at the other 23 internal positions. It is also unlikely that there would not be any changes at these positions on the lineages leading to other game birds whose lysozyme sequences are known, such as chachalaca, turkey, pheasant, chicken, partridge and Old World quail⁶. These considerations, and the conservative nature of the replacements, indicate that the three residues could be forming a structural unit and that replacements at only one or two of the three positions (40, 55 and 91) might not be neutral events. (For a theoretical treatment of non-neutral pathways between functionally equivalent states, see ref. 7.)

TABLE 1 Effect of heating on lysozymes

Lysozyme*	Volume of side chains†	T_m ‡	T_i §	Residual activity (%)
TIS	190	73.9 (0.1)	53.8	75
SIS	167	73.0 (0.1)	53.6	66
TVS	163	71.2 (0.1)	51.1	77
TIT	213	77.5 (0.1)	56.2	80
SVS	140	70.6 (0.6)	51.7	70
SIT	190	75.5 (0.1)	56.3	63
TVT	186	74.5 (0.2)	52.2	76
SVT	163	73.4 (0.1)	54.0	75

* Mutant lysozymes are denoted by the one-letter code for the amino acids at positions 40, 55 and 91, respectively.

† Volumes in Å³ for side chains of residues 40, 55 and 91 (ref. 29).

‡ Thermodynamic transition midpoint based on the decrease in absorbance at 292 nm¹². Samples were 25 µM in lysozyme, 100 mM in potassium phosphate, pH 6.5. The thermal transition is completely reversible for enzymatic activity. Parentheses enclose the standard error of duplicate trials.

§ Temperature required for 50% inactivation of the enzyme in 15 min. To assess the kinetics of inactivation under conditions as nearly physiological as possible, lysozymes were heated in 50 mM sodium bicarbonate buffer, pH 9.0, using the concentrations of lysozyme and ovalbumin found in egg white (0.2 mM and 1.4 mM, respectively)³⁰. Samples were taken at 10 time points over three or more half lives. Enzymes were assayed in parallel. Aliquots were chilled on ice and subsequently diluted 50-fold with ice-cold potassium phosphate buffer, pH 6.5, before assaying (in triplicate) for residual bacteriolytic activity using *Micrococcus luteus* suspensions. Inactivation rate constants were obtained by fitting the data to a first-order model using nonlinear regression analysis. The experiment was repeated at different temperatures to generate a range of inactivation rate constants for analysis.

|| The linear plot of the logarithm of the experimental rate constants of inactivation versus the reciprocal of the absolute temperature (Arrhenius plot) was extrapolated to estimate the rate constant of inactivation at egg incubation temperature (37 °C). This estimated rate constant of inactivation was then used to calculate the residual enzyme activity after one week's incubation.

Lysozyme in game birds is encoded by a single gene⁸ and the probability of simultaneous double or triple mutations in a single gene is vanishingly small⁹. Hence, an evolutionary pathway involving at least three steps and two intermediates (between the TIS and SVT states) probably existed. We have developed an *in vitro* biochemical assay to test whether any of the six

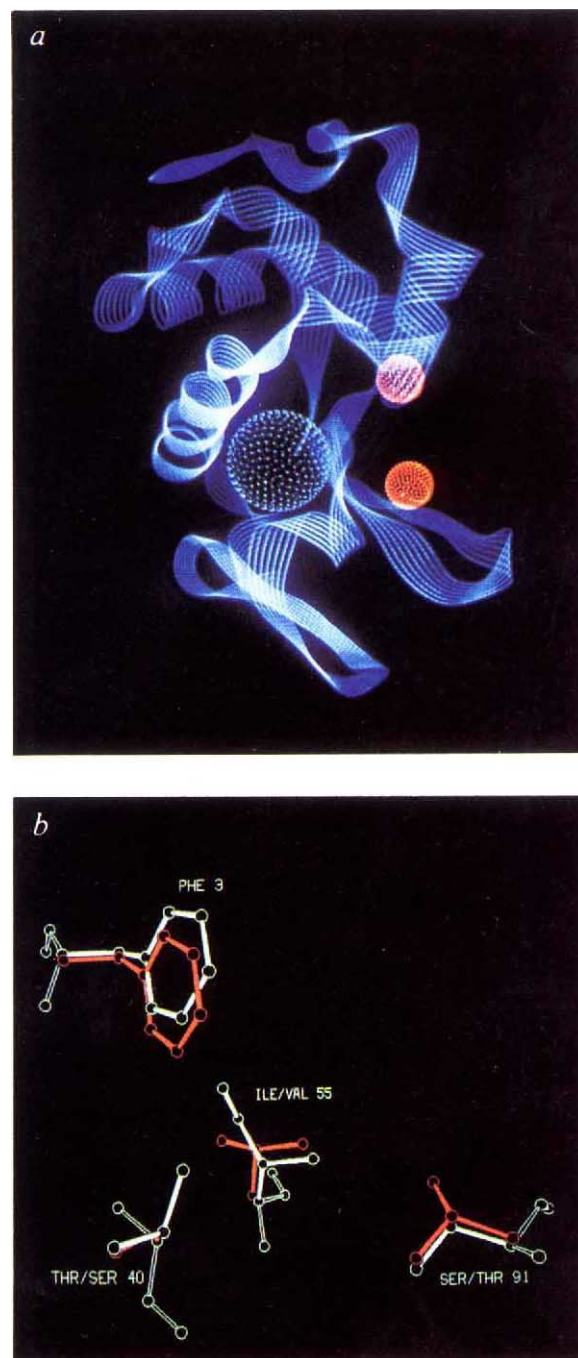


FIG. 1 Two views of the structure of bird lysozyme emphasizing the consequences of changes at an internal cluster of three residues. a, Ribbon structure with the backbone in blue, a large sphere that includes the internal cluster of three residues at positions 40, 55 and 91, and the two catalytic residues at positions 35 (orange) and 52 (pink). b, Ball-and-stick models showing how the residues at positions 3, 40, 55 and 91 are orientated with respect to one another. White side chains represent the TIS structure and the orange ones, the SVT structure. Open sticks connect the backbone atoms (N, C α , C and O) of the TIS structure. These structures are based on X-ray analysis of the synthetic TIS and SVT lysozymes (Table 2).

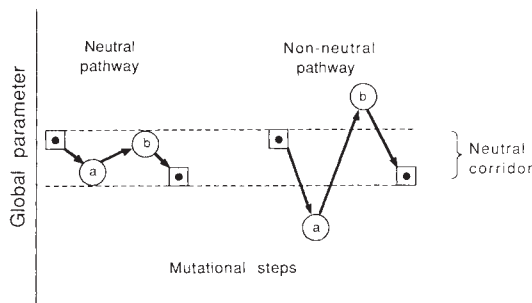


FIG. 2 Two hypothetical evolutionary pathways of protein evolution and the concept of a neutral corridor. Squares denote wild-type states and circles are evolutionary intermediates; arrows depict single amino-acid replacements. The upper dashed line gives the mean value plus two standard errors for an overall property such as thermostability, measured on one of the wild-type proteins; the lower dashed line gives the mean value minus two standard errors for the same parameter measured on the other wild-type protein. The two dashed lines provide a criterion by which a neutral corridor can be defined. In the neutral pathway, both intermediates (a and b) have parameter values within the corridor, but this is not the case in the non-neutral pathway. (The extent of the correlation between a biochemically defined corridor of neutrality and actually being a neutral pathway has still to be established.)

possible three-step pathways between these two states could have been neutral; this tests whether the intermediates are functionally equivalent *in vitro*. Using site-directed mutagenesis of the complementary DNA for chicken lysozyme^{10,11}, we constructed all possible intermediates (SIS, TVS, TIT, SVS, SIT and TVT) in the evolutionary pathway between TIS and SVT. The test makes use of the idea that the two wild-type states (TIS and SVT in this case) define a corridor of neutrality with regard to the overall properties of the native folded enzyme (Fig. 2), the native state being the chief target of natural selection. Failure to be contained within this corridor may be seen as a violation of the *in vitro* criterion for a neutral pathway.

The property that we were able to measure most accurately for each possible intermediate and the wild-type lysozymes was the thermostability. Thermal denaturation of the six possible intermediates and the two wild types was measured spectrophotometrically¹². The melting temperature (T_m) for the two wild-type lysozymes, TIS and SVT, lies between 73 °C and 74 °C, and the range of T_m values for the possible intermediates is from 70.6 °C to 77.5 °C (Table 1). In each of the possible three-step pathways between TIS and SVT there is an intermediate

whose T_m lies significantly outside the narrow thermostability corridor (73 °C–74 °C) defined by the wild-type states. Therefore, none of the six possible pathways lies within the neutral corridor for thermostability (Fig. 2).

To find whether the thermostability of lysozyme might be under natural selection, we studied irreversible thermal inactivation using the free-thiol trap method¹³. A plot of T_m against the temperature necessary for 50% loss of catalytic activity in 15 min (inactivation temperature, T_i) has a slope of almost unity. The rate constants for denaturation measured at temperatures near T_i under quasi-physiological conditions¹⁴ were extrapolated to 37 °C (Table 1). The results indicate that measurable differences in residual catalytic activity might be manifest during the period of egg incubation. Lysozyme function is important in egg white, and so it is plausible that natural selection could scrutinize the resistance of lysozyme to thermal inactivation or to some other property such as proteolysis by invading pathogens¹⁵.

We have also investigated the activity of lysozyme towards three substrates (*Micrococcus luteus* cell walls, glycol chitin and 4-methylumbelliferyl chitotrioside), none of which is encountered naturally by bird lysozymes. Small differences are evident among the six variants and the two wild-type lysozymes: the range of activity is always less than twofold for a given substrate and depends on the nature of the substrate; there is no relation between thermostability and enzyme activity. All pathways between the two wild-type states involve intermediates that lie outside the neutral corridor.

An interesting finding is that there is a strong correlation of T_m and T_i with the combined volume of the side chains at positions 40, 55, and 91 (Table 1; Fig. 2): addition of a methyl or methylene group raises both the T_m and T_i by ~2 °C. To establish the physical basis for this correlation, an X-ray crystallographic study of the lysozymes was undertaken. Seven of the eight synthetic lysozymes form tetragonal crystals suitable for X-ray analysis under the same conditions as those used for the natural enzyme¹⁶; but SVS crystals are too small. Diffraction data were collected by oscillation photography to 1.8 Å resolution (Table 2) and the mutant structures were refined^{17,18} to achieve an estimated coordinate accuracy of ~0.15 Å. The structural changes associated with these mutations are surprisingly small, given their location buried inside the structure (Fig. 1b). Removal of the γ -methyl group of Thr 40 results in a rotation of Phe 3 about its $C\alpha$ - $C\beta$ bond, which repositions the benzyl group to occupy part of the cavity left by the vacating methyl group. Changing Ser to Thr at position 91 introduces a new methyl group that partially fills another cavity in the wild-type enzyme, and causes a slight rotation of residue 55 about its $C\alpha$ - $C\beta$ bond. The ability of the chicken lysozyme structure to accommodate this extra methyl group without strain could explain the enhanced thermostability of the TIT variant.

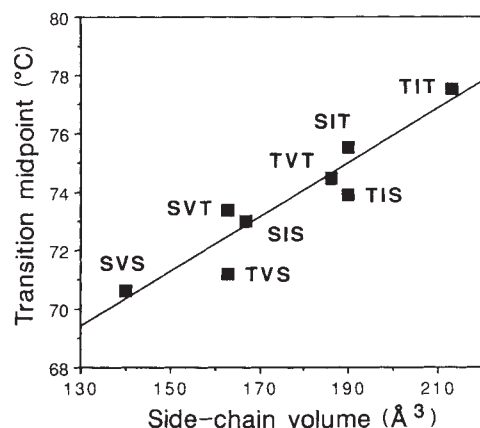


FIG. 3 Dependence of thermostability of lysozyme variants on the combined volume of the side chains at positions 40, 55 and 91.

TABLE 2 X-ray results for seven synthetic lysozymes

Synthetic lysozyme	Resolution (Å)	Residual (%) [*]	Unique reflections	Deviation of bond lengths [†]	Deviation of bond angles [‡]
TIS	1.7	15.2	10,276	0.019	2.23
SIS	1.8	16.6	8,878	0.017	2.41
TVS	1.8	16.0	10,527	0.018	2.47
TIT	1.8	14.0	8,336	0.016	2.21
SIT	1.8	15.7	8,707	0.016	2.14
TVT	1.8	15.2	10,080	0.017	2.43
SVT	1.8	15.3	7,990	0.016	2.43

^{*} Percentage of information in the diffraction patterns that was not explained by the three-dimensional model.

[†] Root-mean-square deviation of bond lengths from ideal lengths, in Å.

[‡] Root-mean-square deviation of bond angles from ideal angles, in degrees.

Differences in electron density associated with the exchange of Val for Ile at residue 55 are limited to the side chain at that position. In all cases, detectable shifts in the backbone atoms are confined to the region of the mutation and do not exceed 0.4 Å. There is no evidence of hinge motion in any of the variant structures. Superposition of all the mutant structures reveals that the SVT lysozyme, in which all three amino acids are altered, differs most from the TIS form (Fig. 1b). The X-ray results therefore indicate that the variations in thermostability among the synthetic lysozymes are due mainly to differences in the packing of side chains inside the protein.

It is widely believed that increasing the density of packing and interior hydrophobic contacts of side chains in a protein will stabilize it against thermal or chemical denaturation, a hypothesis consistent with results from experiments in which large polar side chains have been replaced by smaller ones^{19–21}. On the other hand, substituting small buried side chains with larger ones seems to introduce enough strain into the folded protein to offset any stabilization gained from the extra hydrophobic and van der Waals interactions^{19,21,22}. Examples of thermostabilization as a result of increased hydrophobic packing or replacement of polar with hydrophobic residues^{25–27} are relatively rare^{19,23,24}. The correlation shown in Fig. 3 and Table 1 reveals that three of the 40, 55, 91 triplet variants are more thermostable than the wild-type enzymes. Examination of the structure²⁸ of chicken (TIS) lysozyme shows that the largest cavity is located near Ser 91. The γ -methyl group introduced by the Ser 91 $\rightarrow$ Thr replacement partially fills this cavity without introducing unfavourable van der Waals' contacts: this explains the enhanced thermostability of the TIT variant ($T_m = 77.5^\circ\text{C}$). Our results show therefore that it is possible to increase thermostability by simple hydrophobic packing.

In conclusion, two indirect criteria of neutrality have been presented. We suggest that candidates for non-neutral pathways of protein evolution should violate both of these criteria. One is phylogenetic, and requires that intermediate states be observed in nature at a set of interacting residues; the other requires that the macromolecular properties of synthetic intermediates on the pathway lie within the bounds defined by the wild-type proteins. As the replacements at positions 40, 55 and 91 in game-bird lysozymes violate both criteria, the natural three-step pathway between the SVT and TIS states makes it a candidate for a non-neutral process. We have also demonstrated a relation between thermostability and hydrocarbon packing, which we have partially explained using high-resolution X-ray analysis. The observation that the most thermostable enzyme is not selected from the TIS $\rightarrow$ SVT pathway explored by the evolutionary process indicates that such thermostability is not necessarily advantageous, probably because other important features may be compromised. □

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Resemblance of actin-binding protein/actin gels to covalently crosslinked networks

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THE maintainance of the shape of cells is often due to their surface elasticity, which arises mainly from an actin-rich cytoplasmic cortex^{1,2}. On locomotion, phagocytosis or fission, however, these cells become partially fluid-like. The finding of proteins that can bind to actin and control the assembly of, or crosslink, actin filaments, and of intracellular messages that regulate the activities of some of these actin-binding proteins, indicates that such 'gel-sol' transformations result from the rearrangement of cortical actin-rich networks³. Alternatively, on the basis of a study of the mechanical properties of mixtures of actin filaments and an *Acanthamoeba* actin-binding protein, α -actinin, it has been proposed that these transformations can be accounted for by rapid exchange of crosslinks between actin filaments⁴: the cortical network would be solid when the deformation rate is greater than the rate of crosslink exchange, but would deform or 'creep' when deformation is slow enough to permit crosslinker molecules to rearrange. Here we report, however, that mixtures of actin filaments and actin-binding protein (ABP), an actin crosslinking protein of many higher eukaryotes, form gels rheologically equivalent to covalently crosslinked networks. These gels do not creep in response to applied stress on a time scale compatible with most cell-surface movements. These findings support a more complex and controlled mechanism

underlying the dynamic mechanical properties of cortical cytoplasm, and can explain why cells do not collapse under the constant shear forces that often exist in tissues.

The kinetics of actin-filament assembly⁵ makes it difficult to control the number and length of actin filaments, factors that affect the rheology of polymers^{6,7}. This, coupled with the extreme length of pure actin filaments, presents serious problems for rheological studies⁸. In our experiments we therefore used actin filaments assembled in the presence of the actin-binding protein,

plasma gelsolin. Gelsolin determines the number and length of actin filaments, because it is a powerful nucleator of actin assembly in the presence of micromolar calcium³. Gelsolin binds to the 'barbed' ends of actin filaments decorated with myosin head fragments, and blocks exchange of monomers with that end. Actin filaments 'capped' by gelsolin are therefore more stable than native actin filaments, because addition and loss of monomers at the 'pointed' filament end is extremely slow, and annealing of filaments is prevented^{9,10}.

Figure 1a shows how actin-gelsolin filaments crosslinked by the high relative molecular mass (M_r) F-actin crosslinking protein (ABP)¹¹ respond to oscillating shear forces over a range of frequencies. In contrast to the reported properties of actin/ α -actinin mixtures, the elastic shear modulus G' (a measure of the amount of shear force needed to achieve a given deformation) of actin-ABP gels is constant over a range of frequencies for which actin/ α -actinin showed significant changes. This implies that there are no molecular motions in ABP-crosslinked gels that can dissipate mechanical energy within 100 to 0.01 s, and therefore that ABP-crosslinked filaments are immobilized

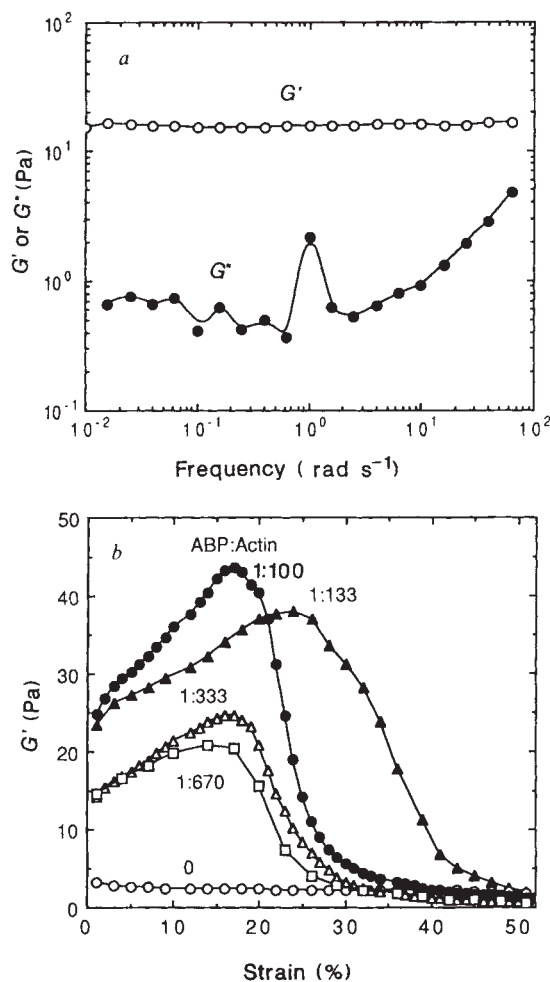


FIG. 1 a, Dynamic storage (G') and loss (G'') shear moduli measured at 1% shear strain over a range of frequencies for 1.6 mg ml^{-1} actin polymerized in the presence of gelsolin and ABP at molar ratios relative to actin of 1:200 and 1:133, respectively. b, Strain dependence of G' measured at $\omega = 1 \text{ rad s}^{-1}$ for 1.6 mg ml^{-1} actin-gelsolin (200:1) filaments polymerized with various amounts of ABP. Measurements were initially made at low strains 90 min after initiation of polymerization, and the maximum strain of the oscillations was increased in successive measurements.

METHODS. Dynamic shear moduli were measured with a Rheometrics RFS8500 instrument at 20°C as described previously⁸. ABP was isolated from smooth muscle-rich human uterine leiomyoma by Wang's method for purification of chicken gizzard filament²⁵. Human uterine smooth muscle ABP, in contrast to chicken gizzard filament²⁶, functionally resembles rabbit macrophage and human platelet ABP (P.A.J., S.H., J.L. and T.P.S., unpublished results). Actin²⁷ was purified from rabbit skeletal muscle and dissolved as G-actin in a solution containing 2 mM tris(hydroxymethyl)aminomethane, 0.2 mM CaCl_2 , 0.2 mM ATP, 0.2 mM 2-mercaptoethanol, pH 7.6, and polymerized at 20°C in the presence of various molar ratios of ABP and human plasma gelsolin²⁸ by addition of 2 mM MgCl_2 and 150 mM KCl. Polymerization occurred between the plates of the Rheometrics instrument and periodic measurements of G' were performed during polymerization to confirm that G' was roughly constant during the time needed to perform frequency- or strain-dependent measurements.

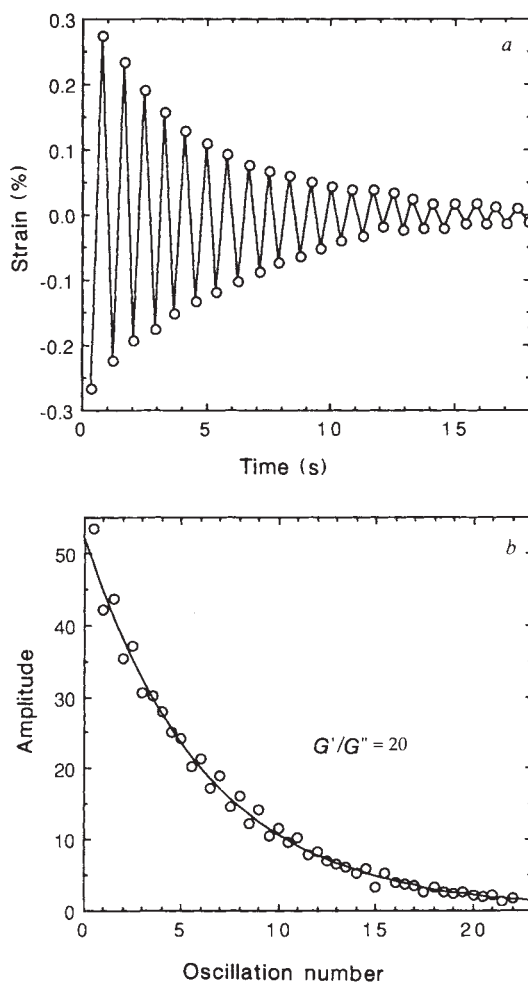


FIG. 2 a, Free oscillation of an actin-gelsolin-ABP gel measured in a torsion pendulum after momentary displacement of the pendulum arm at $t=0 \text{ s}$. The principles of this method and the description of the apparatus are given in refs 7, 17 and 18. Composition of the actin sample was similar to that described in the legend to Fig. 1. Total actin concentration, 2.0 mg ml^{-1} ; actin:gelsolin:ABP ratio was 200:1:1.5. b, Absolute values of successive maxima plotted on an arbitrary scale and the best-fit of a single exponential to these data. The ratio G'/G'' (the inverse of the loss tangent $\tan \delta$) is calculated from the relation $\tan \delta = (\Delta/\pi)\{1/(1+\Delta^2/4\pi^2)\}$, where Δ is the exponential decay constant¹⁸.

over at least 100 s. The elastic storage modulus G' at low frequencies is much greater than the loss modulus G'' , a measure of the amount of mechanical energy dissipated during the oscillation. A large G' -to- G'' ratio at low frequencies is often cited as a criterion for a material to be called a gel. Our findings contrast with results reported by Zaner¹² for actin-ABP mixtures, which also showed frequency-dependence of G' and a low G' -to- G'' ratio. As previously discussed, the instrumentation used in that study probably causes shear-induced rupture of F-actin, as evidenced by low absolute values for G' (ref. 13), which are 10–100-fold higher in more recent studies of F-actin^{8,14–16}, and 200 times greater for 2 mg ml⁻¹ F-actin/ABP (this report).

The mechanical response of actin gels depends on the magnitude of the deformation (quantified as strain) and the rate of deformation. Figure 1b shows how the shear modulus varies with strain for a series of samples containing short actin filaments in the presence of various concentrations of ABP. At all strains, actin-gelsolin samples without ABP have a very low shear modulus. The value of G' increases as the amount of added ABP is increased. When ABP-containing samples are deformed to successively larger strains, the shear modulus at first increases, a phenomenon known as strain-hardening, consistent with the expectation for networks of rigid rods¹⁷. At larger strains, G' decreases abruptly to values equal to those of samples without ABP. The strain-dependence of G' for ABP-containing samples is very similar to that of F-actin without gelsolin⁸. The abrupt

decrease in G' at large strains has been attributed to filament breakage, which seems also to occur in ABP-crosslinked gels, because once deformed beyond a critical strain, these gels do not recover their large initial shear moduli at low strains (data not shown).

The large ratio of elastic storage (G') to viscous loss (G'') of ABP-crosslinked gels shown in Fig. 1a is also manifest in free oscillations induced by a momentary shearing impulse applied to the sample, using an instrument and method¹⁸ independent of that used to obtain the data in Fig. 1. Figure 2 shows a typical damped oscillation of an ABP-crosslinked gel of short actin filaments. The magnitude of G' calculated from the frequency of oscillation (Fig. 2a) and the G' -to- G'' ratio calculated from the rate of exponential decay of amplitude (Fig. 2b) confirm the results shown in Fig. 1.

The slowest molecular motions of polymer networks are generally studied by creep experiments in which a constant shear stress (force per unit area) is applied to a sample and the resulting deformation (shear strain) is measured. Figure 3 compares the creep behaviour of short actin-gelsolin filaments in the presence and absence of ABP. When deformed by a relatively small stress, the actin-gelsolin sample shows a large initial strain, followed by a slow, steady increase in strain, characteristic of sols, including viscoelastic sols (Fig. 3a). When the stress is released, part of the strain is recovered, consistent with some energy being stored elastically by this sample, but there is a

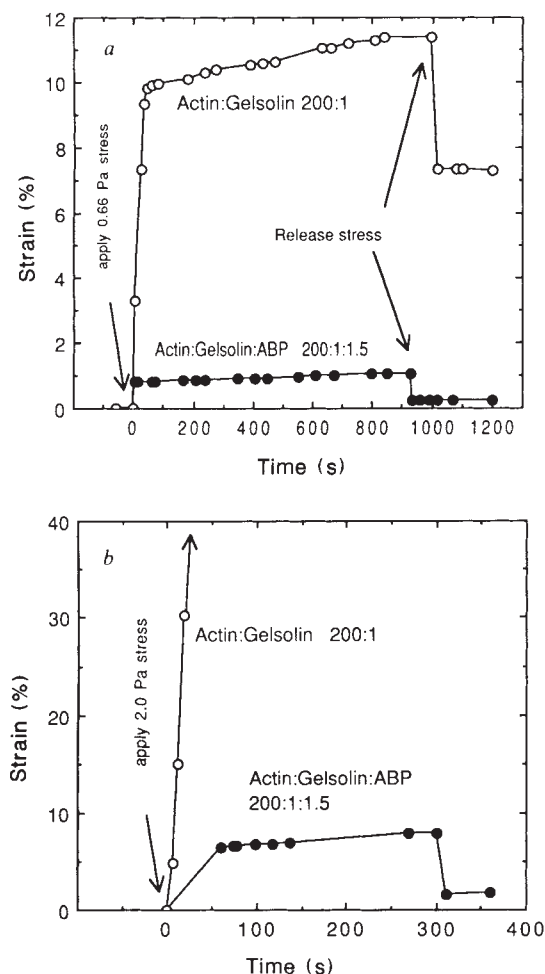


FIG. 3 Time course of shear strain (creep) after imposition of a constant shear stress of 0.66 Pa (a) or 2.0 Pa (b) using the torsion pendulum in a static mode. The shear stress was released at the time indicated by the arrows. The composition of the actin samples is as described in the legend to Fig. 2.

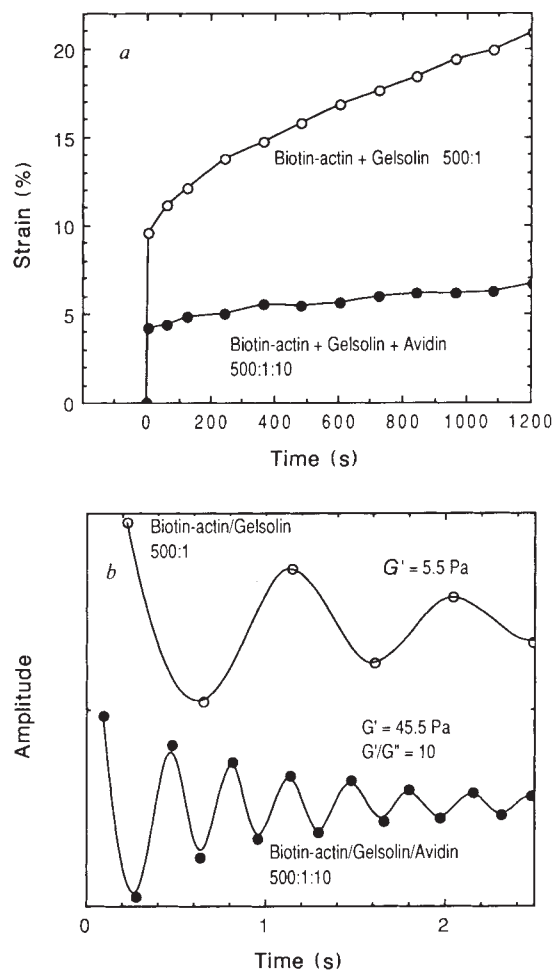


FIG. 4 Shear creep (a) and free oscillations in the torsion pendulum (b) of gels composed of 2 mg ml⁻¹ actin (50% labelled with biotin-maleimide) and gelsolin with or without avidin at molar ratios as shown. The shear moduli were calculated from the frequency and damping of the oscillations¹⁸.

large unrecovered strain, again characteristic of a viscous fluid. By contrast, a sample with the same filament length, but containing ABP, shows a much smaller initial strain, and nearly complete recovery of the strain after removal of stress, characteristic of a viscoelastic solid or gel. In this kind of measurement, which is less perturbing than conventional oscillatory experiments, Zaner also found little creep for ABP-actin¹². The slight increase in strain that is observed is likely to be due to filament breakage between crosslinks, because it is also seen with avidin-crosslinked biotin-actin gels described below (Fig. 4b), but the very slow release of ABP from the filaments cannot be completely ruled out. The ability of ABP to inhibit the shear deformation of short actin filaments is more evident when larger stresses are applied. Short actin filaments flow nearly as a viscous liquid when a critical shear stress is exceeded (Fig. 3b). But the presence of ABP limits the deformation to a small strain that changes very little with time and is largely recovered when the stress is removed.

If the effect of ABP is to induce stable crosslinks between actin filaments, and if the rheology of actin polymers is mainly a function of their concentration, length and degree of crosslinking, then only the stability of the crosslink, and not the exact chemical nature of the bond holding filaments together, should affect viscoelastic properties. To test this hypothesis, we labelled a portion of the actin monomers with biotin by a method that does not affect their incorporation into filaments¹⁹. Crosslinks were introduced by addition of avidin, a tetrameric protein complex with four very high-affinity binding sites for biotin²⁰. Because the dissociation constant of avidin-biotin bonds ($K_d = 10^{-15}$ M) approaches that of covalent bonds, avidin should be an efficient crosslinking agent for biotin-labelled actin filaments. Avidin-crosslinked gels formed from short biotin-labelled actin filaments show lightly damped free oscillations similar to those observed using ABP as the crosslinker (Fig. 4a). The magnitude of G' and the $G'-to-G''$ ratio are similar to the values obtained with ABP-crosslinked gels. Moreover, avidin, like ABP, also greatly inhibits the shear creep of short biotin-actin filaments, resulting in both the initial deformation and slow creep being less than those for short actin filaments (Fig. 4b). That some creep occurs, indicates that actin filaments rupture, consistent with the low tensile strength of single filaments²¹. Avidin-crosslinked biotin-actin gels also show slight strain-hardening at low strains, and rupture above a critical degree of deformation, at strains similar to those observed with ABP-crosslinked gels (data not shown). This result provides further evidence that breakage of actin filaments, rather than dissociation of weak interfibrillar bonds is responsible for the abrupt irreversible decrease of G' observed at moderate strains.

The results presented here do not invalidate the possibility that a dynamic crosslink-exchange mechanism⁴, which has precedents in synthetic polymer chemistry^{22,23}, also operates in the cytoplasm of some cells, but they do show that dissociation of such crosslinks would not lead to cytoplasmic flow if the network also contains a protein such as ABP. It is noteworthy that the frequency dependence of rigidity observed by Sato *et al.*⁴ required α -actinin/actin ratios of the order of 1:15, and many crosslinks per filament. Molar ratios of ABP to actin capable of preventing creep of the actin gel in our study were, by contrast, less than 1:133, and there is sufficient ABP in at least some cells to achieve such ratios throughout the cortical cytoplasm²⁴. The material properties of ABP-crosslinked actin gels allow the cell to be a tough elastic body when subjected to a variety of stresses, while allowing for cytoplasmic flow if the crosslinks are actively detached or the filaments are severed either by mechanical forces or by the activation of specific proteins such as gelsolin. □

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GENERAL

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References should be numbered sequentially as they appear in the text, followed by those in tables and finally those in the figure legends. Reference numbers apply only to papers published or in the press, and a different number should be given to each paper cited. All other forms of reference (including unreferenced abstracts) should be included in the text as personal communication, manuscript in preparation or preprint (with number and institution where appropriate). Text should not be included in the reference list. References should be abbreviated according to the *World List of Scientific Periodicals* (fourth edition, Butterworth, London, 1963–65). The first and last page numbers should be cited. Reference to books should clearly indicate the publisher and the date and place of publication.

Abbreviations, symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided as much as possible and, when used, defined. Editors will shorten words if necessary. In case of doubt, SI units should be used.

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Acknowledgements should be brief. Grant numbers and contribution numbers are not allowed.

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Is non-isotopic *in situ* hybridization finally coming of age?

P. Lichter and D.C. Ward

With recent refinements in non-isotopic *in situ* hybridization methods a plethora of new applications are beginning to emerge.

IN SITU hybridization techniques have been used widely to visualize specific DNA or RNA sequences in preparations of chromosomes, single cells or tissue sections. Initially, the nucleic acid probes were labelled isotopically and detected posthybridization by autoradiography. Over the past few years, however, non-isotopic techniques have become increasingly popular and are being applied to a broad spectrum of interesting biological and clinical problems (Table 1).

In part, this can be attributed to several factors: (1) probes can be modified with reporter molecules (for example, biotin)

TABLE 1 Applications for non-isotopic *in situ* hybridization

Research	Clinical
Gene mapping	Clinical cytogenetics
Gene expression	Prenatal diagnostic
Genome evolution	Carrier status disease genes
Developmental biology	Cancer diagnostics
Tumour biology	Infectious disease diagnostics
Microbiology and virology	Biological dosimetry
Nuclear topography	
Mitosis/meiosis	
Replication timing	
Cell sorting	
Somatic hybrid cell analysis	

both enzymatically and chemically and the detection of bound probes — generally mediated by reporter binding proteins (for example, avidin) — can be achieved in different ways to suit specific experimental needs. Colourimetric assays can be used for studies using transmission, phase-contrast and Normarski optics; fluoro-chrome-tagged probes can be visualized by fluorescence microscopy and quantitated by photon counting devices; heavy-atom compounds, such as colloidal gold, can reveal probe localizations at the electron microscope level; (2) hybridization signals can be more precisely localized than isotopic signals captured by an emulsion overlay, allowing an analysis at much higher spatial resolution and with improved qualitative and quantitative features; (3) non-isotopic detection methods are considerably faster than autoradiographic procedures and in some cases, such as detection via chemiluminescence, can provide even greater sensitivity; (4) non-isotopic probes are chemically stable and are not subject to special disposal requirements; (5) different reporter/detection systems can be used to

facilitate the analysis of several probes simultaneously. To date, reporter group/detection systems have been described for probes labelled with biotin, digoxigenin, dinitrophenol, bromodeoxyuridine, acetylaminofluororene, mercury, sulphone groups, and antibodies for the specific visualization of DNA/RNA hybrids have been prepared. The direct coupling of fluorophores to probe molecules has also been accomplished; (6) a variety of amplification procedures can be applied to enhance sensitivity; (7) digital imaging microscopy can provide further improvement in signal detection and the application of image processing technology provides additional enhancement of signal-to-noise ratios; (8) suppression hybridization techniques, which inhibit hybridization signals from the highly repetitive sequence elements present ubiquitously in the complex genomes of most eukaryotes, facilitate the direct use of probes of any desired genetic complexity, thereby bypassing the requirement for time-consuming subcloning to generate unique sequence probes.

Here, we present a brief overview of some of the recent technical developments and highlight some of the emerging applications. Because of space limitations, comprehensive citation of all the pertinent literature is not possible. Additional relevant publications may be obtained by referral to the bibliography sections of the publications cited below and to those articles comprising the recent memorial issue to Sam Latt in *Cytometry*¹.

Gene mapping

Two major applications of non-isotopic *in situ* hybridization (NISH) that have received considerable attention of late are gene mapping and interphase cytogenetics, the former because of the Human Genome Initiative and the latter because of its potential clinical utility. Although NISH was initially perceived to lack the sensitivity required for routine application to the physical mapping of complex genomes, several groups have reported the visualization of probes ≥ 1 kb long using either conventional microscopy²⁻⁶ or digital imaging microscopy⁷⁻⁹. Probes of considerably greater genetic complexity have a high probability of containing interspersed repetitive sequences, usually

widely distributed throughout the genome, which will result in non-specific hybridization signals. However, protocols designed to suppress these unwanted signals with appropriate competitor DNAs have been defined¹⁰⁻¹², and thus one can obtain highly specific delineation of the genome regions from which the probes have been derived.

Suppression hybridization has been used to map sequences cloned in yeast artificial chromosomes, cosmid, phage and plasmid vectors. With cosmid probes ~90 per cent of the target sequences in cell populations can be delineated by fluorescence, and on metaphase chromosomes spreads more than 80 per cent exhibit specific signals on both chromatids of both chromosome homologs¹³. As virtually every metaphase spread is fully informative, by counting only signals present in parallel on both chromatids as true signals, statistical analysis for precision mapping is greatly reduced and more sequences can be mapped per unit time.

While the use of smaller probe sizes reduces the number of informative metaphases to 20–50 per cent, thereby increasing analysis time, both the sensitivity and throughput are superior to isotopic detection methods. Chromosome assignment is not problematic as several conventional chromosome banding methods, for example, quinacrine, Hoechst 33258, bromodeoxyuridine, and DAPI, as well as hybridization banding¹³ are fully compatible with probe detection by fluorescence. Hybridization signals from two probes can be spatially resolved on metaphase chromosomes when the probes are only several hundred kilobases apart, but a minimum of 1–2-Mbp separation is required to permit their physical order to be established¹⁴.

To further improve the spatial resolution of gene mapping, co-hybridization studies with closely spaced probes from a single genomic region have been performed on interphase nuclei¹⁵. Measurements of the distances between multiple pairs of probes after *in situ* hybridization to methanol/acetic acid-fixed flat nuclei demonstrate a fairly linear relationship between physical distance and genome order over the range from 30 kb up to about 1 Mbp. The combination of metaphase and interphase nuclear mapping,

particularly using multiple probes simultaneously, offers the opportunity to physically order genomic DNA segments with a resolution presently achieved by gel electrophoretic methods and provides a new bridge to interrelate physical and genetic linkage information.

Interphase cytogenetics

The ability to analyse single genes or specific chromosomal regions in interphase nuclei is being exploited in a variety of interesting ways. Some of the strategies employed in these interphase cytogenetic studies are depicted schematically in Figure 1. The fact that the DNA constituting an individual chromosome in a eukaryotic nucleus occupies a discrete intranuclear domain permits the direct quantitation of gene or chromosome numbers given an appropriately specific probe. Complex probe sets, such as a pool of clones from a single chromosome library, have been used under suppression hybridization conditions to decorate, or 'paint', individual chromosomes in a highly specific manner. Composite chromosome 21 probe sets and individual cosmid clones have been used to demonstrate trisomy 21 in interphase cells^{6,12,16}, providing a potential route for the rapid diagnosis of Down Syndrome (Fig. 1a).

Other chromosome-specific probes, often clones of the aliphoid DNA repeat family, are being used to detect numerical chromosome changes in nuclei of cells in specimens from other inherited diseases as well as neoplasias of both hematopoietic cells and solid tissues¹⁷⁻²³. Because of the high efficiency of NISH with cloned probes, deletion of a specific gene or chromosome subregion can also be readily detected in interphase cells (Fig. 1b), with considerably greater ease than quantitative Southern blotting, and viral genome integration sites can be enumerated^{24,25}.

Novel modalities for detecting multiple chromosomal translocations and other types of chromosomal rearrangements in interphase cells of patient origin are also being tested for clinical applications. These range from visualization of specific oncogene rearrangements (Fig. 1c and d) in leukaemias and lymphomas, sensitive detection of chromosome fragmentation due to radiation exposure and the identification of genomic DNA fragments that flank or span specific translocation break points (Fig. 1e and f) that occur in certain types of human tumours.

Cell biological applications

Numerous fundamental questions about the organization and function of both nuclear and cytoplasmic processes can be explored by NISH. All facets of gene expression are amenable to analysis. Indeed, the study of mRNA expression levels has been a major application of *in situ* hybridization for many years. It has

been only recently, however, that an increasing interest in defining the nuclear organization of genes and the processing

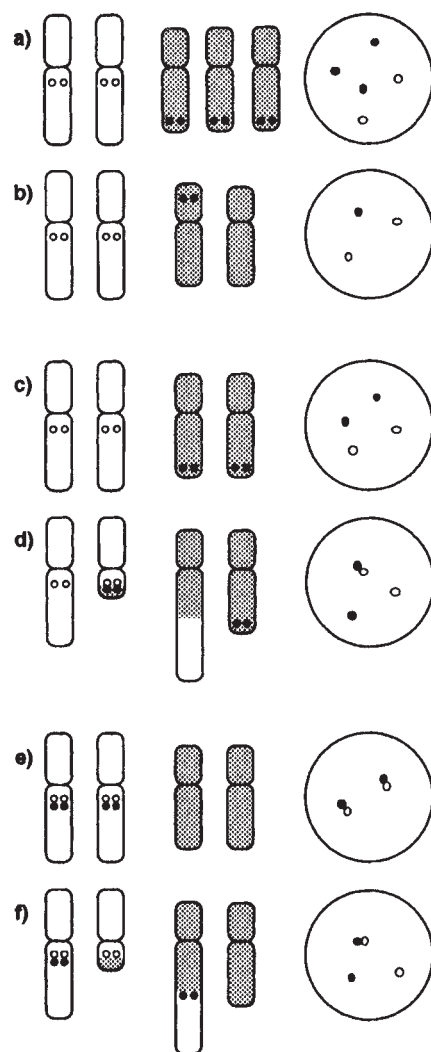


FIG. 1 Schematic illustration of how genomic features on metaphase chromosomes can be detected in interphase nuclei by non-isotopic *in situ* hybridization (NISH). In each lane, two metaphase chromosomes of a particular karyotype are shown as white and shaded chromosomes. Two different signals, as produced by NISH, are shown as white and black circles. Signals in interphase nuclei are shown on the right side of each lane. Note the number or locations of signals. *a*, Detection of trisomic chromosomal material. *b*, Detection of submicroscopic deletions or loss of a single gene. *c* and *d*, Identification of a specific translocation that brings sequences of different chromosomes together. Compare normal case (*c*) and translocation (*d*). *e* and *f*, Characterization of a chromosomal break point or of break-point sequences. In the normal cell (*e*), two signal pairs are seen, whereas in the case of translocation (*f*), one signal pair and two separate signals are visible. Correspondingly, a single clone spanning the break point will give three versus the expected two signals in cells with translocated chromosomes. If the interphase cells are in G2-phase of the cell cycle, all signals are visible as doublets versus the singlets shown in this scheme for G1-phase nuclei.

and transport of their transcripts has evolved²⁶. The ability to visualize newly replicated genes as a pair of intranuclear doublets rather than a pair of single hybridization signals should also facilitate studies on their replication timing during S phase.

With the advent of the confocal laser scanning microscope for optically sectioning cells, hardware and software for computer-assisted three-dimensional image reconstruction, and the broad availability of gene- or chromosome-specific probes, it is now feasible to study intranuclear topography in some detail. An understanding of the spatial arrangement of chromosomes and genes in cells of different developmental lineages may provide new insights into possible relationships between intranuclear location and biological function. Indeed, the initial studies to date support the notion that the chromatin architecture of the nuclear is far from randomly organized²⁷⁻³⁰.

The potential uses of non-radioactive *in situ* hybridization techniques in basic and clinical research will expand still further with the continued refinement of detection techniques, coupled with improvements in optical instruments that will allow the simultaneous visualization of a greater number of target sequences in the same specimen. □

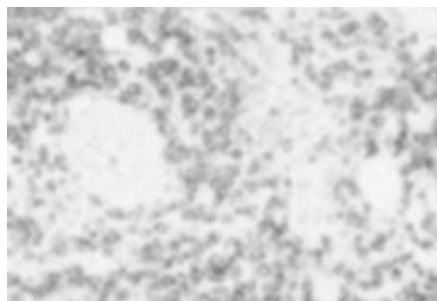
Peter Lichter, Department of Human Genetics and David C. Ward, Departments of Human Genetics and Molecular Biophysics and Biochemistry, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA. For more information, fill in reader service number 100.

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Analytica '90 in preview

Visitors to next week's Analytica '90 trade fair in Munich, West Germany will be able to see a comprehensive selection of the latest equipment for biochemical and instrumental analysis for all fields of application.

HISTOPATHOLOGISTS studying the immuno-histochemical aspects of lymphoid tissue may be interested to learn that Dakopatts has a new **polyclonal antibody**, rabbit anti-human T cell, CD3 (*Reader Service No. 101*). The polyclonal anti-CD3 peptide antibody can be used to detect normal and neoplastic T lymphocytes on paraffin-embedded tissue sections, says Dakopatts. The antibody is affinity-isolated and stains human T cells in both the cortex and medulla of the thymus and in peripheral lymphoid tissues. The antibody is compatible with a variety of staining techniques,

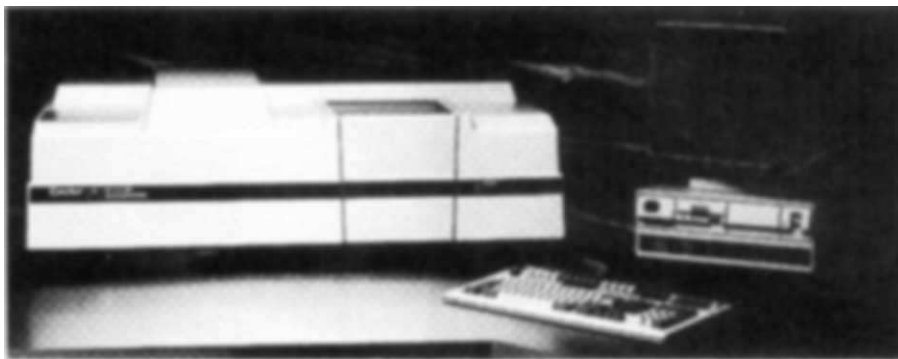


T-cell lymphoma stained for CD3 using rabbit anti-human T cell, CD3.

including peroxidase anti-peroxidase, alkaline phosphatase anti-alkaline phosphatase, and avidin-biotin methods. Visit Dako in hall 5, stand B10.

To meet the needs of toxicologists, pharmacologists and forensic scientists, Sigma has issued a free 1990 **forensic chemistry catalogue** (*Reader Service No. 102*). The 56-page catalogue features an expanded line of controlled substances, deuterium-labelled compounds for use as internal standards in selected-ion monitoring systems, drug standards for use as chromatographic or other analytical standards, pseudonarcotics and related substances, relevant books and instrumentation. Sigma will be in hall 1, stand B11.

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Cary 5: spectrophotometry in the UV/visible/NIR range.

Specs: UV/visible/NIR

Adding to the existing Cary range, Varian, exhibiting in hall 7, stand C17, has announced the availability of the Cary 4 and 5 **UV/visible/NIR spectrophotometers** (*Reader Service No. 103*). At the heart of the system is a new double monochromator, providing improved signal-to-noise ratios and stray light and high-resolution performance standards, says Varian. Cary 4 and 5 spectrophotometers also feature non-measurement phase stepping (NMPS), which produces no peak shift or suppression with changes in scan speed. When performing a wavelength scan with the NMPS system, the gratings remain stationary during the sample and reference phases and move only during the non-measurement phase. The UV/visible/NIR Cary 5 can scan in the 175–3,300-nm wavelength range, while the UV/visible Cary 4 scans up to a 900-nm wavelength. Both instruments also feature extensive software capabilities. Prices start at \$45,000 (US).

The capabilities of the Gilford Response II UV/visible scanning spectrophotometer, available from Ciba Corning Diagnostics, have been extended to include a **thermoprogamming routine** (*Reader Service No. 104*). Using the six-cell Peltier-controlled holder, thermal melting curves and denaturation/renaturation curves of nucleic acid or protein can be produced. The user can program the heating and cooling of up to five 1.5-ml samples and one reference over the 0 to 100 °C temperature range, with an accuracy of ± 0.1 °C. Applications software programs allow absorbance data to be collected at 0.1-, 0.5- or 1.0-°C intervals, with data displayed as absorbance against temperature in graphic or tabular formats. The T_m of nucleic acids can be determined by manual extrapolation, or automatically using a first-derivative

calculation. By entering the buffer calculation, the percentage G–C content can be determined. Renaturation kinetics can also be calculated by both linear and non-linear curve-fitting methods. The spectrophotometer runs all the usual UV/visible methods, such as wavelength and time scans, over a 190–900-nm range. See Ciba Corning (Kleinfeld stand) in hall 9, stand A3.

Analysis: IE/HPLC

The IS3500 **HPLC system** from LDC Analytical is designed to combine the performance versatility of the spectroMonitor 5000 photodiode array detector with the precise flow stability of the constaMetric 3200 fluid metering pump (*Reader Service No. 105*). The spectroMonitor 5000 provides complete spectral information on an LC separation, with a sensitivity of 0.0005 AU, says LDC. The detector can store up to 300 scans in its internal memory, or can



For complete data manipulation and integration, use LDC Analytical's IS3500 HPLC system and software.

transmit 10 scans per second to a PC. The constaMetric 3200 provides high solvent delivery precision from 10 μ l to 10 ml min⁻¹, ensuring, says LDC, reproducibility of peak retention times. Used in conjunction with the ThermoChrom PDA data system software, IS3500 provides total data manipulation and integration.

The IS3500 HPLC system with software but without a PC costs DM45,900 (FRG). See LDC in hall 2, stand C30.

Econo-System, on view in hall 3, stand B14, is the latest integrated **low-pressure chromatography system** from Bio-Rad, which offers programmable binary gradients and peak collection at 254/280 UV-detection (*Reader Service No. 106*). The system consists of a pump that has a 0.1–2.0 ml min⁻¹ range, UV detector, integrator module for the pump and detector, fraction collector and data recorder. The system is suitable for cold-room use and can be fitted with columns of up to 5-mm ID. The Econo-System supports a full range of separation tech-

niques, including ion exchange, affinity and hydrophobic interaction chromatography. The components making up the Econo-System can be purchased individually for use with existing laboratory equipment, or as an integrated system.

Molecular miscellany

Using the A.S.A.P. genomic DNA isolation kit, Boehringer Mannheim says users can purify intact, high molecular weight genomic DNA in three hours or less, without the need for organic solvents (*Reader Service No. 107*). The \$150 (US) A.S.A.P. kit contains prepared columns that can be used to purify genomic DNA from a variety of prokaryotic and eukaryotic sources, eliminating the need for phenol and chloroform extraction and caesium chloride gradients. According to

cess (*Reader Service No. 108*). Special features of the gel dryer include a variable temperature control, which facilitates short drying periods, and a fine vacuum-setting control (manometer controlled). Drystar is available in three models, each differing in the size of the drying area. When necessary, the drying pad and rubber sealing pad can easily be replaced. Prices for the Drystar gel dryers, which can be seen in hall 2, stand B25, range between DM2,033 and 2,860 (FRG).

Imaging hardware

For the simultaneous measurement and observation of electrochemical reactions, EG & G has introduced a **scanning tunneling microscope (STM)** called MicroView (*Reader Service No. 109*). MicroView combines state-of-the-art imaging hardware and computer technology with a built-in potentiostat and electrochemical cell. This allows the user directly to observe and measure a surface in air or solution at up to atomic resolutions and as an electrochemical reaction occurs. Using the quantum-mechanical phenomenon of 'tunnelling', whereby electrons appear to bore through barriers such as air or water, MicroView electrochemically stimulates a sample *in situ* and measures the resulting reaction. A real-time graphical image of the reaction site is displayed on the video monitor. MicroView scans a 12 × 12-μ area and, typically, a 128 × 128 element scan takes about two seconds, says EG & G. Three-dimensional images of the surface topography take only 12 seconds to process. Over two-dozen image processing techniques are available and up to four images can be displayed simultaneously. EG & G will be in hall 5, stand B9.

Joyce-Loebl will be launching its new C1000 **analogue camera** at Analytica, which features a dual-function mode that is switchable between the traditional 512 × 512 pixels and a new 1,024 × 1,024 scanning format (*Reader Service No. 110*). Filling a gap in the existing market, the C1000 offers a high-resolution image at a cost only marginally greater than that of traditional analogue devices. The camera takes four pictures sequentially, which are then combined into a single image by the image analyser. Initially, the camera will be introduced as part of Joyce-Loebl's Gemini electrophoresis workstation, but its use is to be extended to autoradiography and chromosome applications. See the C1000 in action in hall 7, stand B29. □

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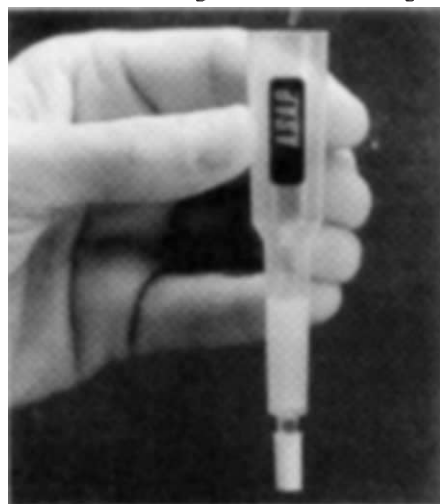
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The Drystar **gel-drying system** from H. Hölzel of Germany is designed to provide a vacuum-tight seal during the drying pro-



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